FUNGI ASSOCIATED WITH STORED WHEAT GRAIN IN AUSTRALIA: ISOLATION, IDENTIFICATION AND CHARACTERISATION

by

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Declaration

The work described in this thesis was undertaken while I was enrolled as a student for the degree of Doctor of Philosophy at Murdoch University, Western Australia. I declare that this thesis is my own account of my research and contains as its main content work that has not previously been submitted for a degree at any tertiary education institution.

The following people assisted with some aspects of the research described in this thesis: Professor Giles Hardy provided expertise in morphological identifications; Mrs Diane White and Ms Banafshe Safaiefarahani assisted with the DNA extractions and preparation for sequencing (Chapters 3 and 4); Associate Professor Treena Burgess and Dr Kirsty Bayliss helped with the phylogenetic analyses (Chapters 3 and 4); Associate Professor Mike Calver contributed to the statistical analyses; Professor YongLin Ren and Mr Bob Du assisted in the extraction, measurement and analysis of volatile organic compounds (Chapter 5) and Mr Ernie Steiner of the Department of Agriculture and Food WA conducted the gamma-irradiation of all grain required for the experiments.

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Abstract

Fungal contamination of stored grain is a major concern because it can cause economic losses and potential human and animal health issues. The aims of this study were to: determine the effects of temperature, grain moisture content and length of storage on survival of fungi associated with stored wheat grain in Australia; compare the efficacy of a range of methods for isolating, identifying and characterizing fungi in stored wheat grain in Australia; and assess the potential of using volatile organic compounds for detecting fungi associated with stored wheat grain in Australia.

It was demonstrated that fungi could be isolated from apparently healthy wheat grain for up to six months when stored at recommended or slightly higher than recommended moisture contents and temperatures. Spoilage fungi associated with wheat grain from across Australia were isolated and identified and included the genera *Alternaria*, *Aspergillus*, *Aureobasidium*, *Cladosporium*, *Drechslera*, *Fusarium*, *Mucor*, *Nigrospora*, *Penicillium*, *Rhizopus*, *Setosphaeria*, *Stemphylium*, *Ulocladium*, *Epicoccum* and an unidentified species from the Hypocreales. The genus *Eutiarosporella* was also recorded and a new species, *Eutiarsporella pseudotritici-australis* sp. nov. described. The most frequently isolated genus was *Alternaria*.

Next generation sequencing was used to identify fungi that were not isolated using traditional methods and detected the presence of the human pathogens *Cryptococcus macerans* and *Cryptococcus victoriae*. It was also demonstrated that that some fungal species produce volatile organic compounds including cyclooctasiloxane hexadecamethyl- (CAS 556-68-3) from *Alternaria alternata*, pentadecane (CAS 629-62-9) from *A. infectoria* and naphthalene (CAS 91-20-3) in grain colonised by *Cladosporium herbarum*.

This research has contributed new knowledge regarding fungal pathogens occurring in stored grain in Australia. When combined with next generation sequencing and measurement of volatile organic compounds the presence of fungi in storage facilities may be determined at an early stage of development, allowing mitigation strategies to be implemented to reduce postharvest loss and ensure the supply of quality grain in Australia.

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Chapter 1: Literature Review

1.1 Introduction

Food security is a global priority (Campbell 2009; Hoffmann 2011; Porter *et al*. 2014). There is an increasing demand for food, yet the global food supply is being challenged by several factors such as changes in the patterns of spatial and temporal distribution of water available for agriculture, temperature patterns and frequency of adverse weather conditions and geographical distribution of pests and diseases (Millar and Roots 2012). Food production requires continued innovation and resilience in farming systems, including better preparedness and improved risk management. In addition, food production must be achieved sustainably, i.e. without affecting agricultural land and natural ecosystems for future generations.

Cereals, including wheat, maize (corn) and rice, are the major staple foods required by the world's population, for both human and animal consumption. It is imperative that cereal production meets the demands of our growing world population, which is estimated to reach 9 billion by 2050 (Porter *et al*. 2014).

Wheat is the largest crop with regards to value, for food consumption and energy production. For example, approximately one quarter of all human energy in the United States is from the consumption of wheat in some form, whilst in Europe it is approximately 50% and in Asia about 75% (Mathew *et al*. 2010).

Wheat is the most valuable cereal crop grown in Australia (Fig. 1.1), and the major wheat producing states are Western Australia (WA), New South Wales (NSW), South Australia (SA), Queensland (QLD) and Victoria (VIC) (Murray and Brennan 2009). Australia is the fourth biggest wheat exporter in the world after the US, Canada, and the European Union (Yang *et al*. 2014). WA is the largest grain producer in Australia, at 40 % of the nation's crop (Yang *et al*. 2014).

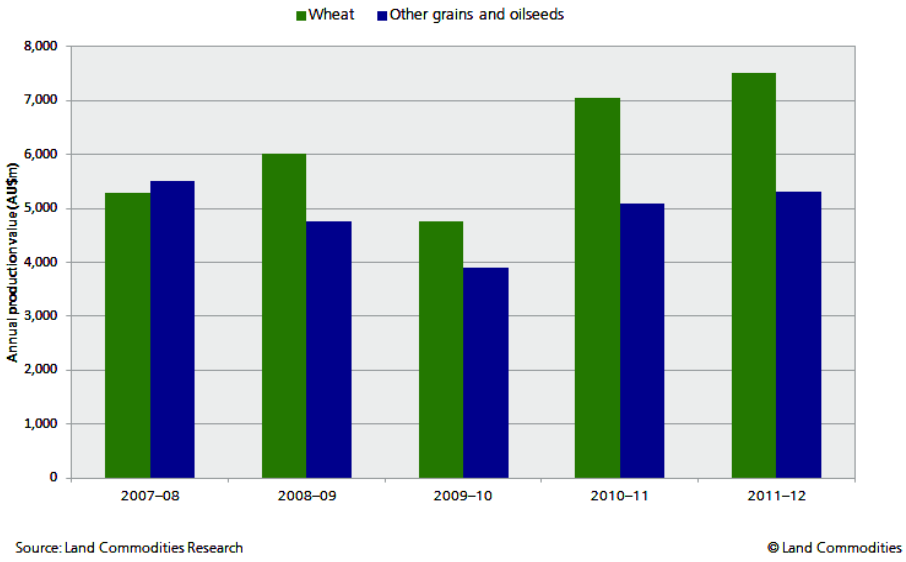


Figure 1.1 Australian wheat and other grain production value from 2007 to 2012
Source: http://www.landcommodities.com/crop-production-in-the-australian-wheatbelt/

Worldwide, cereal production has failed to meet consumption needs and loss due to storage pests is estimated at around 10% to 30% annually (Chelladurai *et al*. 2010). In many developing countries losses of 10-15% in stored products including cereals and legumes are caused by insect and microbial activities (de Lucia and Assennato 1994; Neethirajan 2007). Twenty-five million tons of wheat is lost during different postharvest stages including storage and post-production in Australia, USA, and West Asia. The main biotic factors influencing wheat loss during storage are insects, moulds, birds and rats (Baloch 1999).

Anwar *et al*. (2013) reported that climate change could also have major effects on wheat production in Australia; for example, wheat productivity in New South Wales, valued at $2.85 billion (Yang *et al*. 2014), is extremely sensitive to climate influences such as drought episodes and elevated temperatures (Paterson and Lima 2010). Climate change is having significant impacts on human health, agriculture, settlements and infrastructure, tourism, biodiversity and natural ecosystems and will continue to do so. It also may have impacts on plant breeding, plant diseases and mycotoxins in Europe, Australia and Africa (Garrett *et al*. 2006; Boken *et al*. 2008; Chauhan *et al*. 2008, 2010; Miraglia *et al*. 2009; Paterson and Lima 2010).

In Australia, climate change has indirectly impacted land values in areas of wheat cropping due to increasing temperature and declining rainfall from 1990 to 2011; temperatures are

projected to increase considerably by 2100 (Chakraborty *et al.* 1998). Extreme weather events in Australia such as droughts, floods, tropical cyclones and severe storms (Yang *et al*. 2014) can also have negative effects on wheat production, particularly due to erratic rainfall or high temperatures. These factors can influence infection of crops by fungi that grow and produce mycotoxins (e.g. aflatoxins). Changes in CO_2 concentrations as a result of climate change also impact on wheat production. Anwar *et al*. (2007) forecast the impacts of predicted climate change and showed that wheat yields were likely to be impacted negatively in south-eastern Australia, with a decline in the median wheat yield by about 25% together with an increased prevalence of pests and diseases, as well as soil degradation due to temperature change. Global warming may also produce other ecological changes that might impact agricultural production (Coakley *et al*. 1999). For example, the appearance of new diseases due to modifications in the distributional range, temporal activity and community structure of pathogens. Further, phenology and growth conditions of the hosts will be altered (Jeger *et al*. 2007; Lonsdale and Gibbs 1996), thereby affect production and yield.

Whilst food production is an important part of food security, less consideration has been given to postharvest losses of food supplies, especially losses related to a reduction in food quality due to increased activity of biological organisms such as pests and diseases, yet spoilage accounts for significant losses of edible grain. Indeed, the postharvest loss of cereals is highest during storage after harvest (Magan *et al*. 2003). Magan *et al*. (2011) demonstrated that postharvest losses are caused by a wide variety of biotic and abiotic factors. These include mould, insects, mites and the key environmental factors of water and temperature. The interactions between these factors affect the dominance of fungi, especially mycotoxigenic species such as *Fusarium culmorum, Aspergillus ochraceus* and *Penicillium verruscosum*. Therefore, minimising postharvest losses caused by these pathogens is an effective way to improve agricultural income (Tefera *et al*. 2011). The purpose of this review is to assess the main causes of postharvest losses in cereal grains, and the methods used for the isolation, identification and characterization of fungi associated with stored wheat grain.

1.2 Common postharvest problems

Postharvest losses commonly occur when stored grain is attacked by insects, mites, rodents, birds, and microorganisms (Neethirajan *et al*. 2007). Such infestations cause a reduction in product quality and economic loss (Birck *et al*. 2003; 2006).

Invertebrate pests of grains include more than 600 beetle species, 70 species of moths and about 355 species of mites, all of which cause quantitative and qualitative losses during grain storage (Rajendran 2002). The most common stored grain insect species are *Sitophilus oryzae* (lesser grain weevil), *S. zeamais* (greater grain weevil), *Oryzaephilus surinamensis* (saw-toothed grain beetle), *Tribolium castaneum* (red flour beetle), *Rhyzopertha dominica* (lesser grain borer) and *Cryptolestes ferrugineus* (rusty grain beetle) (Beckett 2011). Contamination is due to the presence of eggs, larvae, larval exoskeletons, pupae and pupal cases, cocoons and mature insects. Faecal material from insects is another source of contamination (Christensen and Kaufmann 1969). Temperature and moisture conditions in grain storage facilities can be manipulated (see Section 1.4) to reduce the population growth rate of insect pests (Driscoll *et al*. 2000).

In Australia, storage pests can reduce the quality of grain, impacting on the ability to export grain. The major common pests of wheat grain presently found in Australia include *Tribolium castaneum* and *Rhyzopertha dominica* and the warehouse beetle, *Trogoderma variabile* (Niu *et al*. 2012). However, grain producers in Australia have good management practices to maintain freedom of grains from pests during storage (see Section 1.4.3).

Rodents, birds and even bats can also become pests if they enter grain storage areas. They consume stored grains and pollute the grain with their dung during feeding and/or use the facilities as refugia. Large numbers of birds can damage storage structures and can pose health risks. For example, large winter flocks of 2,000 starlings in Alberta, Canada can consume 4.5×10^{-4} to $9.0\text{x}10^{-4}$ tons of feed in a month and pollute or destroy an extra 0.498 to 0.998 tons of feed through their waste (Weaver and Petroff 2005). Controls include protecting grain from pests using vermin proof construction and suitable pest control programs as well as ensuring storage silos and other containers are clean and disinfected before grain is unloaded (Bricknell *et al*. 2006). It is essential to continue to develop technologies for stored grain management, to prevent pests entering the wheat and to control them when they do.

1.2.1 Stored grain pathogens

In terms of microorganisms, fungi and their associated secondary metabolites known as mycotoxins are of high concern in grain shipments or storage facilities due to the production of mould, odours, the presence of microbial 'hot-spots', and the production of secondary metabolites which can lead to subsequent poisoning of food and animal feed, thus negatively impacting food safety (Tefera *et al*. 2011).

There are a number of postharvest fungi that can attack and cause damage to grain, and they can be divided into two groups: field fungi and storage fungi (Miller 1995). Field fungi may modify the structure and quality of seeds or grains (Chelladurai *et al*. 2010). These cause damage to the grain before harvest and can generally be detected by routine assessment. In general, field fungi do not occur in storage if the grain is stored at appropriate moisture contents and temperatures (Christensen and Kaufmann 1965). Storage fungi are those that cause damage to grain during storage and usually do not occur at a serious level prior to harvest (Muir and White 2000).

The mycoflora of stored grains predominantly consist of the ubiquitous mould genera *Aspergillus*, *Alternaria*, *Cladosporium*, *Fusarium*, *Mucor*, *Rhizopus* and *Penicillium* (Mathew *et al*. 2010). They are usually introduced into the stored grain as spores in minute quantities during handling and storage. Other microorganisms such as certain bacteria can also colonise grain. These bacteria mainly belong to the families Pseudomonadaceae, Micrococcaceae, Lactobacillaceae and Bacillaceae (Laca *et al*. 2006). In Australia, Europe, and the US *Salmonella* spp., *Escherichia coli*, and *Bacillus cereus* are also present in wheat and flour at low levels (Cicognani *et al*. 1975; Ottogalli and Galli 1979; Spicher 1986; Eyles *et al*. 1989; Richter *et al*. 1993 and Aydin *et al*. 2009). The presence of these bacteria and fungi and their adverse effects can be compounded further by insect activity (Jayas *et al*. 1994) and if the moisture content rises above 13%-15%, wheat moulds begin to develop (Jay 1996). Protection can be achieved by decreasing grain temperature and controlling moisture migration with aeration or by using ambient or refrigerated air (Collins and Conyers 2010; Donahaye *et al*. 1995; Banks and Fields 1995; Fields 1992) (see Section 1.4 for optimum storage conditions in Australia).

Mycotoxins produced by some fungi cause a large number of diseases annually, from liver and esophageal cancer, acute toxicosis, immune suppression, and can also stunt growth in

children (Wu *et al*. 2011). The majority of infections of animals (e.g chronic aflatoxicoses) on farms are caused by mycotoxins being present in poor quality feed (Zain 2011). For example, Aflatoxin B1 is highly toxic and is a potent carcinogen to both humans and animals. Fumonisin B1 (FB1) is produced by *Fusarium moniliforme* and can cause equine leucoencepha-lomalacia (Kellerman *et al*. 1990) and porcine pulmonary edema (Harrison *et al*. 1990); these infections were observed in animals after they had consumed contaminated corn. Very few cases of human disease caused by mycotoxins have been recorded in Australia but have been recorded in animals (Blaney 2007; Tobin 1988; Webley and Jackson 1998). It is important to note that fungi can produce extremely high mycotoxin concentrations in small pockets of grain, which have the potential to contaminate larger amounts of grain at levels exceeding acceptable limits for domestic and export markets (Magan *et al*. 2003).

1.2.2 Pathogens known to occur on stored grain in Australia

In the last 20 years there have been few reports of fungi causing spoilage in grain in Australia (Table 1.1). This most likely reflects the strong regulatory controls present in Australia related to grain storage.

Table 1.1 Fungi reported in stored grains in two states of Australia

Crop	State	Pathogen	References
Wheat	Queensland	*Aspergillus candidus*	Blaney (1986)
		Aspergillus flavus	
		Aspergillus glaucus	
		Mucor sp.	
		Aspergillus ochraceus	Connole *et al*. (1981)
		Aspergillus sp.	Berghofer *et al*. (2003)
		Cladosproium sp.	
		Eurotium sp.	
		Penicillium sp.	
Corn	North Queensland	*Acremonium zeae*	Blaney (1986)
		Aspergillus flavus	
		Aspergillus niger	
		Curvularia sp.	
		Drechslera sp.	
		Fusarium oxysporum	
		Fusarium subglutinans	
		Nigrospora sp.	
		Penicillium sp.	
		Rhizoctonia sp.	
		Trichoderma sp.	
		Fusarium graminearum	Williams and Blaney (1994);
		Aspergillus flavus	Blaney *et al*. (1984, 1986, 2006)
Sorghum	Queensland	*Alternaria*	Blaney and Williams (1991)
		Fusarium	
Wheat	Western Australia	*Alternaria tenuis*	Shipton and Chambers (1966)
		Aspergillus spp.	
		Bipolaris sp.	
		Chaetomium sp.	
		Cladosporium cladosporioides	
		Cochliobolus spicifer	
		Helminthosporium sativum	
		Mucor sp.	
		Malustela aeria	
		Paecilomyces sp.	
		Penicillium spp.	
		Pleospora infectoria	
		Podosporiella verticillata	
		Rhizopus sp.	
		Septoria nodorum	
		Septoria sp.	
		Stemphylium dendriticum	
		Stemphylium sp.	
		Thielavia terricola	
		Ulocladium sp.	

1.3 Detection and identification of fungi in stored grain

1.3.1 Traditional methods

The majority of fungal detection methods in food sources require representative sampling and dissection of the food sample before fungal growth can be detected (Magan and Evans 2000; Paolesse *et al*. 2006 and Schnürer *et al*. 1999). As the techniques used often provide only presence/absence data rather than a direct indication of the extent of fungal invasion, if contaminants are found, the food product may be discarded or downgraded (for animal feed) (Golob 2007). Traditionally, methods for the identification of stored fungi were based on the morphology of cultures and microscopic features such as spores and fruiting structures. Methods for the detection of mould contamination and mould growth using agar media have a number of disadvantages. Counting of colony forming units is slow and not related to actual fungal activity. Representative sampling is also difficult, as this method requires a number of days from isolation to identification and is time consuming and expensive. Furthermore, culture medium preparation, inoculation of plates, colony counting and biochemical characterisation are labour intensive. Currently, there is a strong demand in the market for rapid isolation and identification methods, or other methods that detect the presence of a detrimental microorganism or pest (Paolesse *et al*. 2006). Methods such as molecular tools or the analysis of volatile organic compounds (VOCs) by gas chromatography–mass spectrometry (GC-MS) are particularly promising techniques for grain samples as they can reduce the time required to determine the presence of contaminated and mouldy grain, from days to a few hours or even minutes (Girotti *et al*. 2012; Nicolaisen *et al*. 2014).

1.3.2 Molecular methods

Molecular methods are useful for taxonomic classification, phylogenetic inference and species delimitation and identification (Begerow *et al*. 2010). A variety of molecular methods have been developed to assist and facilitate differentiation between fungal species. These methods include analysis of ribosomal DNA (rDNA) and mitochondrial small subunit (SSU) rDNA sequences (Bensassi *et al*. 2009) and DNA bar coding (i.e. the use of 500–800-bp long DNA sequences to delineate species) (Begerow *et al*. 2010). The internal transcribed spacer (ITS) region is the most commonly sequenced genetic marker of fungi and it is regularly used to answer research questions relating to systematics, phylogeny and

identification of strains and specimens at and below the species level (Begerow *et al*. 2010). Next generation sequencing also has great potential in the molecular identification of fungi when present in small amounts or where species are otherwise difficult to tell apart (Begerow *et al*. 2010). Next generation sequencing platforms are helping to open completely new areas of biological inquiry, including the classification of ecological diversity, and the detection of unknown etiologic agents (Mardis 2008). However, these methods are not always applicable due to the time (a mimimum of several hours), cost and labour required, particularly for individual samples (Girotti *et al*. 2012). Thus there is a need to investigate alternative detection methods for fungal contamination in grain that are both efficient and inexpensive, that may potentially be used on farm during early stages of grain storage.

1.3.3 Chemical and biochemical detection methods

A range of methods can be used for the early detection of volatile organic compounds produced by fungi, other microorganisms or insects in grain (Demyttenaere *et al*. 2003 and Paolesse *et al*. 2006). For example, gas chromatography (GC) or gas chromatography–mass spectrometry (GC-MS) can be used to quantify the key volatile compounds produced in the presence of known spoilage fungi (Schnürer *et al*. 1999). These volatile compounds, if specific to certain fungal genera or species can then be used for the rapid determination of the presence of these fungi in a stored grain sample (Nieminen *et al*. 2008). Detection of moulds in grain can be based on odours from volatiles such as alcohols, esters, ketones, and mono- and sesquiterpenes and aldehydes. For example, Sahgal *et al*. (2007) confirmed that the fungal volatiles 3-octanone, 1-octanol, and 3-methyl-1-butanol produced by *A. flavus*, *A. ochraceus*, *A. oryzae*, *A. parasiticus*, *A. nidulans*, *P. chrysogenum*, *P. citrinum*, *P. funiculosum*, *P. raistricki*, *P. viridicatum*, *Alternaria* spp., *Cephalosporium* spp. and *Fusarium* spp. found in stored barley and wheat could be detected by GC or GC-MS. A broad range of techniques used for the early detection and analysis of a wide range of fungal volatiles produced in cereals are available (Girotti *et al*. 2012; Andreu and Picó 2004; Table 1.2).

Table 1.2 Advantages and disadvantages of various detection techniques for volatiles (Andreu and Picó 2004).

Technique	Advantages	Disadvantages	References
TLC Thin-layer chromatography	An extremely powerful, rapid and an inexpensive separation	Weak fluorescence, low sensitivities and poor recoveries	Betina 1989; Xu *et al*. (2006)
HPLC High performance liquid chromatography	Successfully applied for determination of citrinin in soft wheat, wheat bran, rice, barley, corn	Low reproducibility of chromatographic behaviour on retention time	Xu *et al*. (2006)
LC-UV Liquid Chromatography-UV	Application to virtually any organic solute, regardless of its volatility or thermal stability	Insufficient separation efficiency and selectivity	Andreu and Picó (2004)
GC/MS Gas chromatography–mass spectrometry	High resolving power and ability to resolve individual analytes High sensitivity and selectivity Existence of mass spectrum libraries for screening unknown samples Compositions of both mobile and stationary phase are variable Can be automated and miniaturized (microchip technology) Low price, simplicity, robustness and large linear range Lack of matrix interferences	Inadequate for polar, thermo-labile and low volatility compounds High consumption of expensive, high-purity gases Large amounts of expensive, toxic, organic solvent used as mobile phase	Andreu and Picó (2004); Qiu *et al*. (2014b)

Various extraction methods combined with gas chromatography-mass spectrometry are used for the detection of volatile organic compounds, and include headspace gas chromatography/mass spectrometry (HS-GC/MS) (Colomb *et al*. 2008), purge-and-membrane mass spectrometry (PAM-MS) (Ojala *et al*. 1999; 2001), and proton transfer reaction-mass spectrometry (PTR-MS) (Hansel *et al*. 1995, 1998; Lindinger *et al*. 1998; Fall *et al*. 1999). These techniques combine both qualitative and quantitative approaches in headspace sampling, as sample headspace volatiles are automatically brought directly to the GC, and provide the opportunity for a high number of samples to be processed, and

validated. Headspace solid-phase microextraction (HS-SPME) is a simple and easy technique that can be used for the rapid detection of fungal contamination of food samples, such as cereals, cheeses and other dairy products (Demyttenaere *et al*. 2003). SPME is increasingly being used instead of the traditional and time-consuming methods of isolation and identification of fungi (Qiu *et al*. 2014b). The majority of SPME applications have been performed in combination with GC, where the analytes are thermally desorbed from the fiber into the injector of the chromatograph (Song *et al*. 1998). SPME in combination with GC has a great potential to identify fungi present on wheat grain at early stage of grain colonisation, and it is a convenient alternative method for the analysis of VOCs in wheat.

The sensitivity and selectivity of conventional gas chromatography flame ionization detector (GC/FID) or GC/MS with HS-SPME was sufficient to identify compounds present at trace levels (at levels as low as 10^1 CFU/g barley grain) (Olsson *et al*. 2002). These methods are clearly very sensitive, easy to use, more reliable and more sensitive than traditional methods and the more recent DNA techniques (Begerow *et al*. 2010). Nevertheless, these methods do require significant technical and analytical knowledge. Particularly in the food industry there is a need for more rapid methods to provide sufficient information on the possible presence of pathogens in raw materials and finished food products (De Boer and Beumer 1999).

1.4 Best practice for postharvest storage

Cereal production varies from year to year; therefore, grain storage is important to ensure supply in years of under-production. Grain is also stored (often long term) prior to processing. Correct postharvest storage is crucial to avoid losses in volume, quality and value of the grain (Banks 1999). Farms systems in Australia comprise various on-farm operations, including application of fertilisers and chemicals (herbicides, insecticides, fungicides). Operations are different on each farm.

Best practice for grain storage includes correct storage spaces on farms and during transport and shipping, and management factors that ensure the maximum protection of grain at an optimum quality. Strategies to effectively preserve grain quality include (1) excluding the entry of moisture into grain storages, (2) reducing the temperature at which the grain is stored, (3) adequate pest vertebrate control, and (4) preventing fungal and insect infestations. The major prevention and control tools used for on farm storage systems are

drying, aeration systems and chemical control of pests including application of insecticides and fumigants (Sinha 1995).

1.4.1 Moisture control

Moisture content is one of the most important factors that affects grain quality. This includes ensuring that water cannot enter grain storage facilities, as well as the moisture content of the grain itself. Attention should be given to diverting sources of moisture contamination from grain as early as possible in the supply chain (Magan *et al*. 2003) and by ensuring grains are optimally stored at less than or equal to 12.5% moisture content (Metz 2006). Grain stored at high moisture contents (15 to 16 per cent and at 30°C) is subject to deterioration by microorganisms (Magan *et al*. 2003; Fig 1.2 a,b).

1.4.2 Temperature control

Environmental temperature influences the rate of fungal growth and the production of mycotoxins (Magan *et al*. 2003). During storage, grain temperatures will change slowly over weeks or months, as heat is gained and lost depending on weather and storage conditions (Fig. 1.3). In general, storage fungi grow most quickly at 30°C; however, *A. flavus* grows fastest on moist grain at 45°C and *A. candidus* at 55°C. Consequently, prior to storage, grain temperature should be reduced to 12-15°C (Christensen and Kaufmann 1965). However, grain temperatures are typically between 10°C (a minimum of 5°C) and 35°C (maximum of 40°C) in Australian storage facilities (Wilson 1949; Griffiths and Scientific 1964; White *et al.* 1988).

Cooler grain temperatures can be achieved through aeration of storage facilities through the use of ambient or refrigerated air (Beckett 2011). In Australia, grain cooling by aeration is commonly used for stored grain maintenance and is used for inhibition of insects, control of moisture migration and conservation of grain quality (Driscoll *et al*. 2000). The maximum and minimum temperatures for insect and mould growth in stored grains are outlined in Table 1.3. Above or below these temperatures, insects or moulds are less capable of doing damage and causing problems.

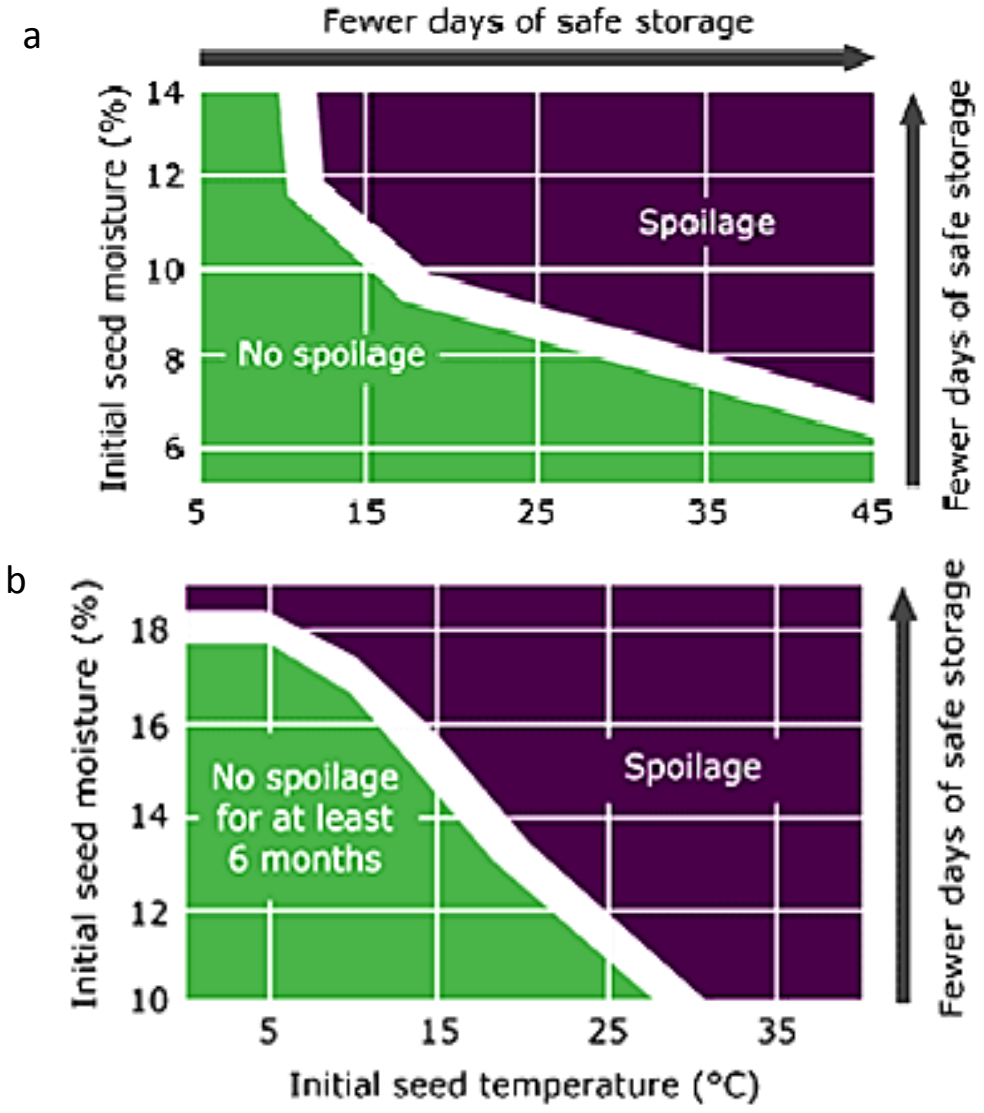


Figure 1.2 The relationship between grain temperature, moisture content and days of safe storage. a. spoilage occurs when initial temperature ranges from 5°C to 45°C with respective moisture from 14% to 6% moisture content, b. spoilage occurs when initial temperature ranges from 5°C to 25°C with respective moisture from 18% to 10% moisture content.(Source:https://www.grainscanada.gc.ca/storage-entrepose/ssg-de-eng.htm

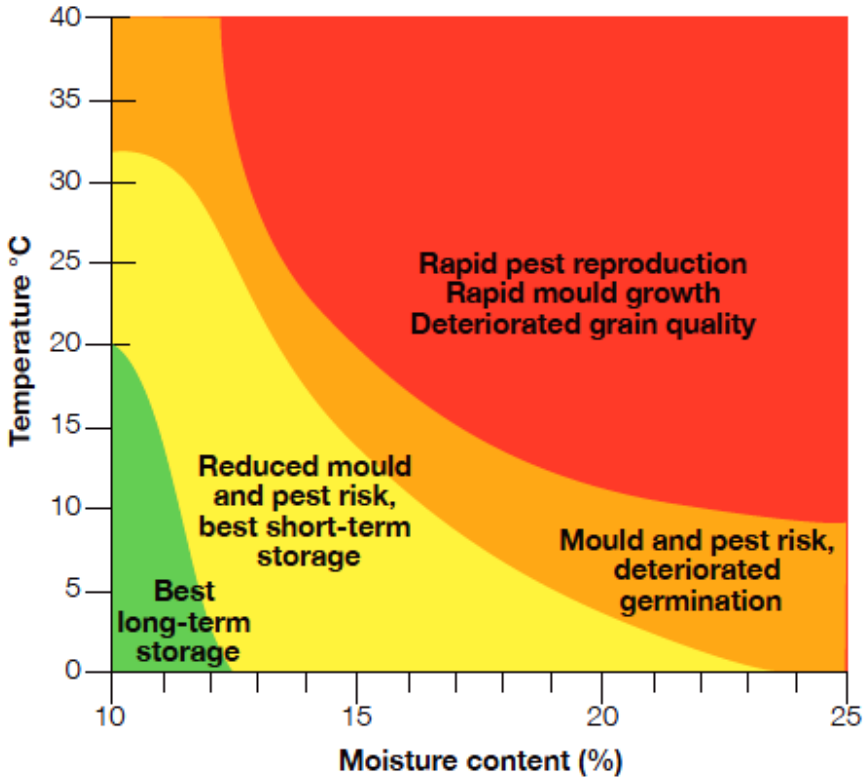


Figure 1.3 Effect of temperature and moisture on stored grain (Source: http://storedgrain.com.au/wp-content/uploads/2013/07/GSFS-7_HighMoistureGrain_2013_LR_Final.pdf).

Table 1.3 The critical temperatures for fungi and insect growth in stored grains

Pests	**Min T (°C) - Max T (°C)**	**References**
Insects		
Rhyzopertha dominica, Sitophilus oryzae, Oryzaephilus surinamensis and *Tribolium castaneum*	15-33°C	Cassells *et al*. (2003); Muir and White (2000); Malaker *et al*. (2008)
Fungi		
Aspergillus penicillioides and *Eurotium repens*	20-30°C	Beckett *et al*. (1994); Driscoll *et al*. (2000)

Min T (minimum temperatures), Max T (maximum temperatures)

1.4.3 Invertebrate control

The most important procedure to prevent insects invading grain storage facilities is to reduce the overall numbers of insects in the grain facility and to prevent the spread of infested grain to other nearby storage structures. To protect grain, sealed storage systems can be fumigated with phosphine and kept at oxygen levels below 2% (Banks and Fields 1995). Importantly, good insect control methods will also effectively control fungal growth, provided the grain is sufficiently dried before storage, and the diurnal temperature fluctuations within storage are minimized (Neethirajan *et al*. 2007).

1.4.4 Microbial control

Mould growth in stored grain is determined mainly by moisture content and how mould interacts with temperature and gas composition. Fungal activity can cause rapid deterioration of grain if incorrectly stored, in particular if there are increases in temperatures, changes in moisture content or changes in gas consumption. The process of drying grain plays an important role in minimizing the adverse effects of spoilage due to fungi. The maximum moisture content for safe storage of wheat is 13% (Kaleta and Górnicki 2013), at a temperature of 20°C (Ramakrishna *et al*. 1993). These values are important to maintain in order to avoid risk of mould infestation. In addition, changes in the concentrations of O_2 and CO_2 in the atmosphere of grain bulks can be significant in modifying fungal colonization during storage. Magan and Lacey (1988) reported that low levels of O_2 effect the germination of *Aspergillus versicolor* and *Penicillium roqueforti* conidia from 75% and 90% to 10% and 0%, respectively. However, appropriate storage conditions should be considered and evaluated, particularly those affecting the quality of stored grain, such as moisture content, temperature and storage time. These are the most significant factors conditioning the development of mycotoxigenic fungi during storage, which are of importance for the management of hazards to human and animal health (Reuss *et al* 1994).

1.5 Thesis Aims

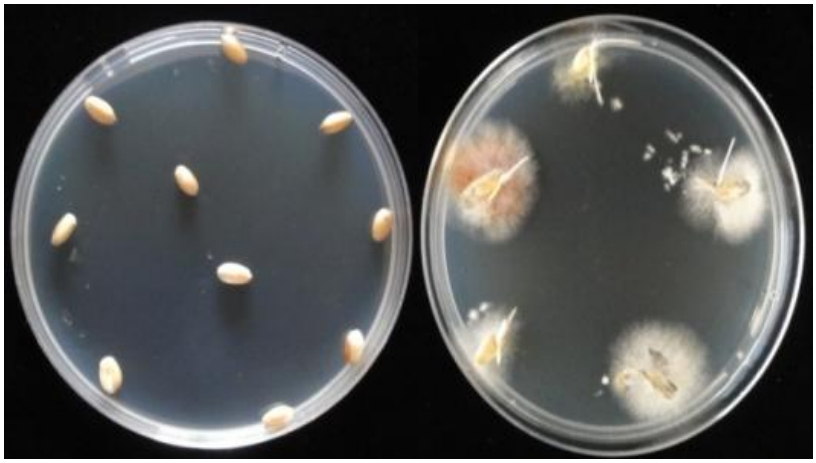
If not stored correctly, wheat can be a favourable host for a range of fungal species that can be detrimental to the quality of the final product. In Australia, the increasingly variable climate, characterized by high rainfall and humidity is likely to increase the incidence of

moulds in stored wheat. This has already occurred: in the 2011 season, due to higher than expected rainfall in the eastern Australian grain growing regions, large quantities of grain was lost or damaged (Yang *et al*. 2014). The objectives of this study were to:

- Determine the effects of temperature, grain moisture content and length of storage on survival of fungi associated with stored wheat grain in Australia;
- Compare the efficacy of a range of methods for isolating, identifying and characterising fungi in stored wheat grain in Australia; and
- Assess the potential of using volatile organic compounds for detecting fungi associated with stored wheat grain in Australia.

Chapter 2: Temperature and grain moisture content stimulate the growth of fungi on healthy stored wheat grain

Source: http://glennpasch.com/wp-



2.1. Introduction

Effective long-term grain storage requires correct management of the grain. In Australia, grain is typically harvested at 12.5% moisture content for long-term (6+ months) storage in sealed silos (Metz 2006). Aeration systems are also used to cool the grain temperature to approximately 20°C, which aids in reducing insect proliferation (Driscoll *et al*. 2000). Temperature cables are useful in detecting "hot spots" where moisture, mould growth and insect infestation may occur. Temperature monitoring is also essential to check the progress of grain cooling during aeration.

Storage fungi can be present on grain at low levels at harvest, and also on grain handling and storage equipment and structures. If fungal growth proliferates it can increase the temperature of the grain, which in turn attracts insects and mites to the warm, humid conditions. In turn, insect respiration will produce further moisture to stimulate more fungal growth (Neethirajan *et al*. 2007). The aim of this study was to investigate abiotic factors contributing to growth of fungi during grain storage. The hypothesis was that a low level of fungal contamination is always present in healthy (no visual symptoms or signs of fungal infection) stored grain, and these fungi can be stimulated to grow depending on the temperature and moisture content at which the grain is stored, and the duration of storage.

2.2 Materials and Methods

Australian Hard White wheat grain was supplied by Cooperative Bulk Handling, Western Australia. All grain appeared healthy with no visual symptoms or signs of fungal infection. Prior to commencement, three replicates of 10 grains each were randomly selected and plated onto potato dextrose agar (PDA; Becton, Dickinson and Company, Sparks, USA) to determine the number of fungi present prior to storage. Grain was then stored at one of three moisture contents (11, 13 or 15%) and one of three temperatures (20, 25 or 32.5°C) for one, two or six months, in the dark. Where necessary, the moisture content was adjusted by placing the wheat in a sealed flask (3L) which was sealed and allowed to eqilabrate to 25°C for three days before the addition of distilled water, and confirmed with an electronic moisture meter (Graintec HE 50 electronic moisture meter, Graintec Pty Ltd, Toowoomba, Australia). The experiment was repeated once. In Experiment 1, 14 g of grains was stored in sealed glass McCartney bottles, whilst in Experiment 2, 200 g grain was stored in in

sealed glass jars (250 ml). The storage container volume was either 250 ml (with plastic caps) or 25ml (with metal lids) and both allowed air exchange.

The headspace of the storage containers was calculated using the following equation:

Headspace (ml) = V_1 (volume of bottle with water) - V_2 (volume of bottle with grain)

There were three replicates for each time/temperature/moisture content combination. Controls consisted of grain sterilised by gamma-irradiation for one week at 30,000 GY. At the end of each time period, 10 grains were randomly removed from each replicate and plated onto PDA and the mean number of these grains that exhibited fungal growth after two days at 25°C was recorded. Mycelial tips were transferred to PDA and colonies identified using using the morphological criteria of Barnett and Hunter (1972), Booth (1971), Ellis (1971), and Nelson *et al*. (1983). Three-way ANOVA using IBM, SPSS Statistic (Version 21) was used to analyse the differences between treatments.

2.3 Results

A total of thirty isolates were isolated at the commencement of the experiments (prior to storage), except from the gamma-irradiated controls where no isolates were obtained. Genera isolated included *Eutiarosporella*, *Drechslera*, *Nigrospora*, *Ulocladium*, *Penicillium*, *Epicoccum*, *Aspergillus*, *Alternaria* and *Rhizopus*, and isolation frequencies were similar in both experiments.

<u>At 11% moisture content</u>

There was no significant ($F_{4, 54} = 0.52$, $P = 0.071$) difference in the ability to isolate fungi from grain at 11% moisture content, stored at any temperature for up to six months in Experiment 1 or at 20°C or 25°C after 1 or 2 months in Experiment 2 (Fig. 2.1a,b). After six months the number of grains from which fungi could be isolated started to decrease at 25°C in Experiment 2 and no fungi were isolated from grain stored at 32.5°C (Fig. 2.1b).

<u>At 13% moisture content</u>

In Experiment 1 there were no significant ($F_{4, 54} = 2.353$, $P = 0.065$) differences in the number of grains stored at 13% moisture content from which fungi were isolated after one or two months (Fig. 2.2a) but after six months less grains exhibited fungal growth at 25°C. In Experiment 2 the number of grains with fungal growth increased after two months months at 25°C but decreased significantly at 32.5°C. After six months, there were

significantly ($F_{4, 54}$ = 5.81, P = 0.0005) less grain with fungi isolated at 20°C and 25°C, with none isolated at 32.5°C (Fig. 2.2b).

At 15% moisture content

There were no significant ($F_{4, 54}$ = 1.50, P = 0.21) differences in the mean numbers of grains exhibiting fungal growth following storage for 1 or 2 months at 20°C and 25°C in both experiments (Fig. 2.3a). By 6 months, the number of grains with fungi decreased at 25°C for Experiment 1. In contrast, no grains had fungal growth in Experiment 2 after six months storage at 20°C and there were significantly fewer grains with fungal growth at 25°C and 32.5°C at the same time period (Fig 2.3b).

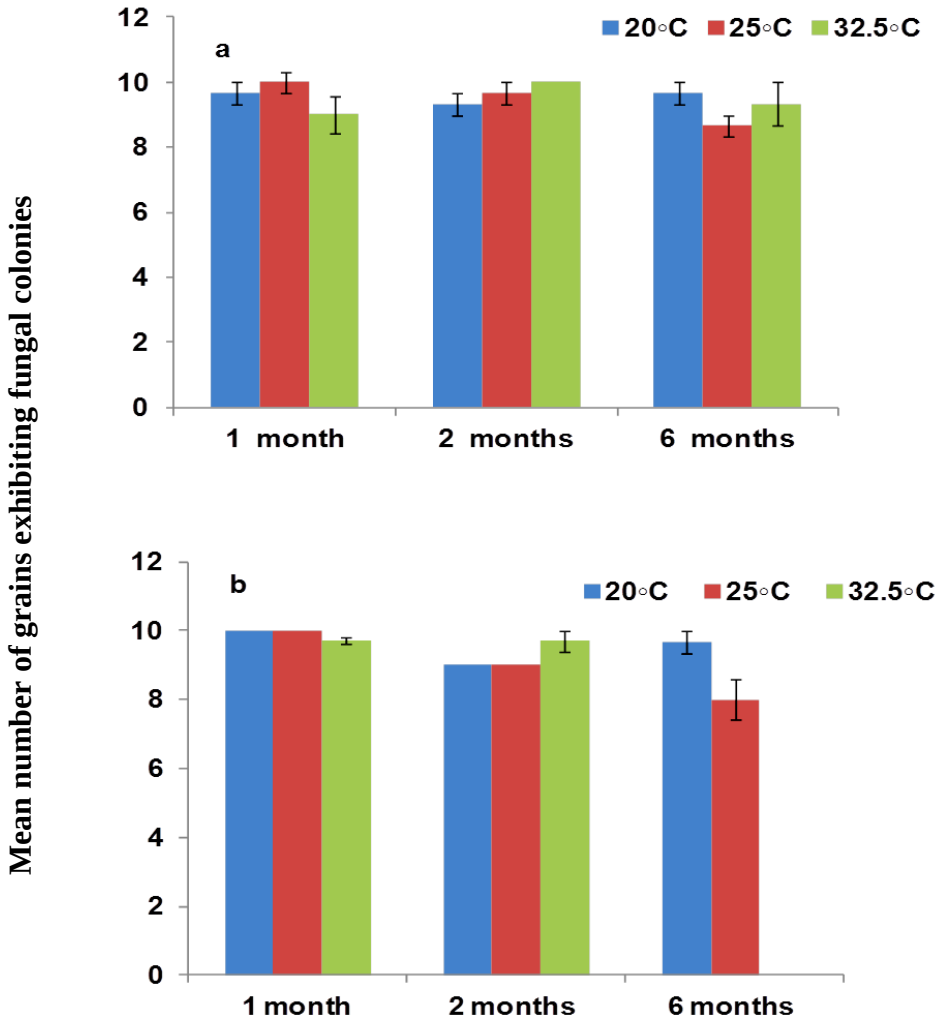


Figure 2.1 Mean number of wheat grains exhibiting fungal growth after being stored at 11% moisture content at 20°C, 25°C, or 32.5°C for 1, 2 and 6 months for a) Experiment 1 (14 g grain) or b) Experiment 2 (200 g grain). Bars represent standard errors of the mean.

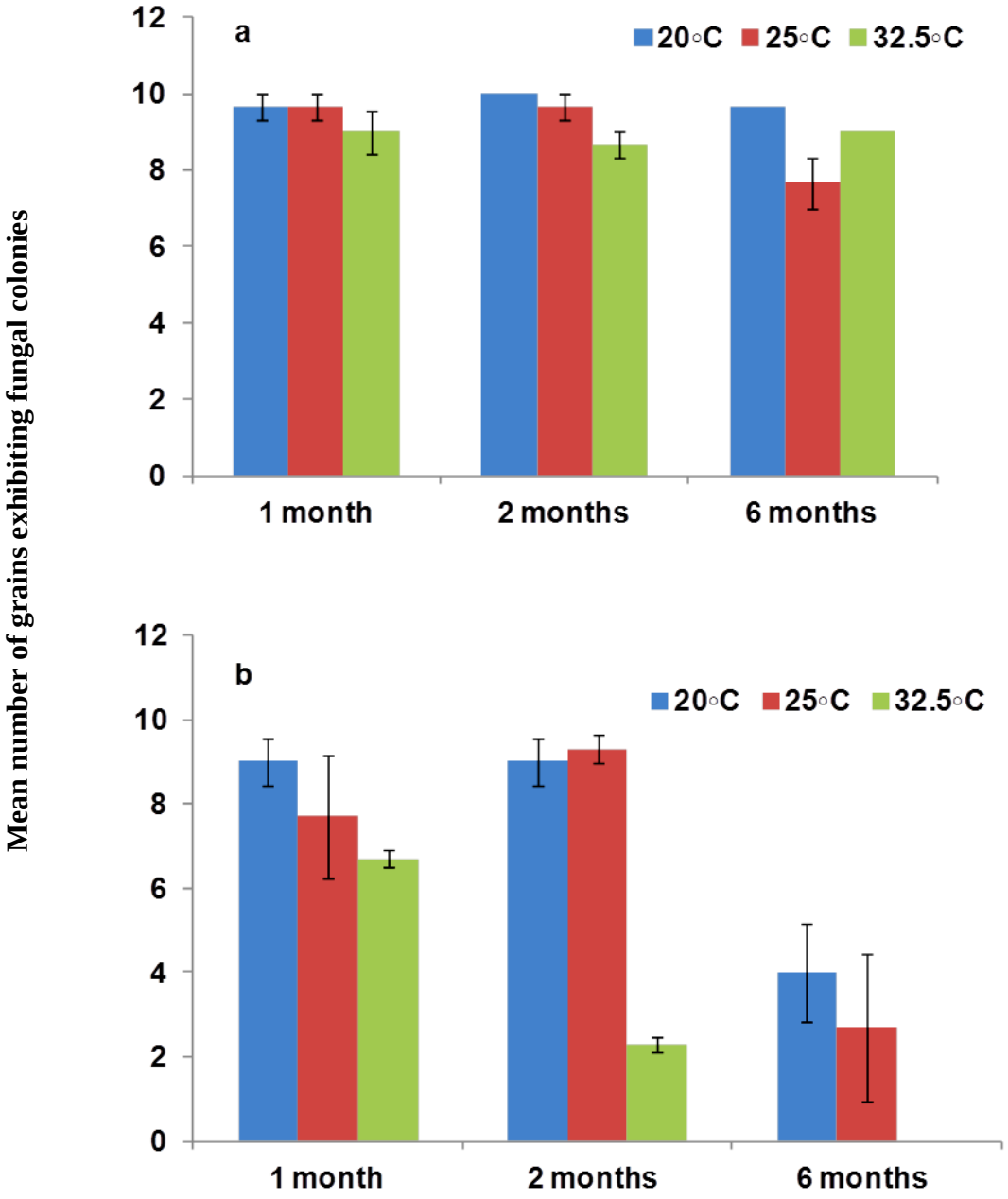


Figure 2.2 Mean number of wheat grains exhibiting fungal growth after being stored at 13 % moisture content at 20°C, 25°C, or 32.5°C for 1, 2 and 6 months for a) Experiment 1 (14 g grain) or b) Experiment 2 (200 g grain). Bars represent standard errors of the mean.

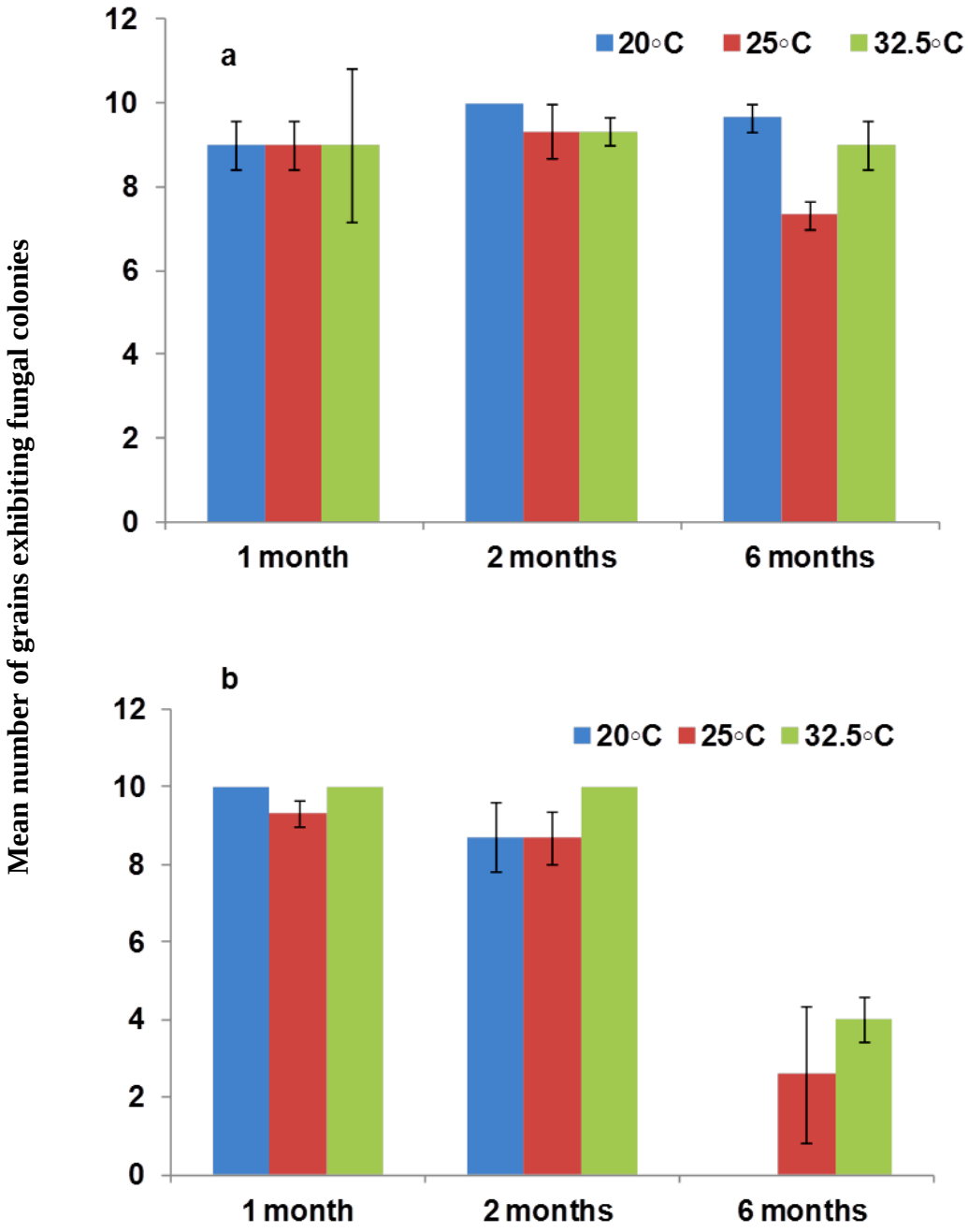


Figure 2.3 Mean number of wheat grains exhibiting fungal growth after being stored at 15 % moisture content at 20°C, 25°C, or 32.5°C for 1, 2 and 6 months for a) Experiment 1 (14 g grain) or b) Experiment 2 (200 g grain). Bars represent standard errors of the mean.

2.4. Discussion

This study demonstrated that fungi could be isolated from apparently healthy (visually free from mould) grain for up to six months when stored at recommended or slightly higher than recommended moisture contents and temperatures. These results are consistent with the hypothesis that a low level of fungal contamination is always present in healthy stored grain. All genera isolated were previously recorded on stored wheat in Australia (Chapter 1, Table 1.1). Therefore, if grain storage facilities are breached and allow ingress of water, it is highly likely that these fungi will proliferate and cause spoilage-associated problems.

The number of grains from which fungi could be isolated generally decreased with increasing storage time, however the number did vary depending on the volume of the stored grain, with significantly fewer isolations from grain stored in the larger storage vessels. This finding is contrary to expectations, as it would generally be expected that a smaller headspace would quickly accumulate CO_2 and deplete O_2, thereby reducing the growth of fungi (Magan and Lacey 1988). Low levels of oxygen (< 0.5 %) affect the germination, sporulation and respiration of fungi during storage, generally leading to moulds progressively disappearing as they are killed by anaerobiosis (Richard-Molard *et al*. 1984). In the current experiments the larger vessels in Expriment 2 had a 10-fold greater headspace volume (120 ml) compared with the smaller vessels (12 ml) in Experiment 1, yet the larger vessels had less fungi isolated over time. It is possible that the unexpected results might have occurred because the larger grain volume produced more CO_2 more rapidly than the smaller grain volume and so depleted O_2 levels more rapidly than relative headspace volumes alone.

The results from this experiment contrast those of Budd (1959), Clarke and Hill (1981) and Burrell *et al*. (1978) who stored wheat under completely airtight conditions in jars and found no fungi could grow in moist grain and that any existing fungi died. The difference between the current experiments and previously published results may be due to the atmosphere and gas exchange within the storage vessels. Moisture content, temperature and concentrations of gases (CO_2, O_2, SO_2 or NH_3) all influence fungal growth during storage, and the interaction between these factors is also important. For example, Magan and Lacey (1988) and Burrell *et al*. (1978) reported that lowering the incubation temperature increased the concentrations of SO_2 or NH_3, both of which can be inhibitory to microbial growth.

In this experiment it is likely that the smaller storage vessels with metal lids and rubber seals may have allowed some gas exchange compared to the larger vessels with plastic lids, which helped to maintain the growth of fungi. Similarly, Muir and Wallace (1973) reported that sealed jars with metal lids may have permitted some gas exchange which lead to an increase in growth of *Penicillium* spp. and *Aspergillus* spp. in wheat stored in their jars.

Another possible explanation for the lower number of fungi being isolated from grain in Experiment 2 could be due to the characteristics of individual fungal species within the storage vessels. Stored grain can be colonized by a range of different fungi that compete for space, nutrients and gas exchange. Marín *et al*. (1998) reported that the competitive species are dependent on the interaction between index of dominance (*Id*), growth rates and environmental conditions such as water activity (a_w,) and temperature. For example, some *Aspergillus* species inhibit the growth of some *Fusarium* species. Further, according to Magan and Lacey (1984), changes in the concentrations of CO_2 or O_2 in stored grain can be important in modifying fungal colonisation by *Aspergillus versicolor* and *Penicillium roqueforti* during storage. In the current study, full identification of isolates was not completed as the focus was on numbers rather than diagnostics, however it is possible that the colonies differed between the two experiments, resulting in different numbers being isolated. Competition among fungi could also have affected the number of isolations from grain, however further work is needed to confirm this.

Differences between field and storage fungi may also play a role in determining which species will dominate a stored grain ecosystem under a particular set of environmental factors. During long-term storage, fungi of the genera *Aspergillus* and *Penicillium* ("storage flora") progressively replace the "field flora"such as *Fusarium* and *Alternaria* over a period of several months (Karunakaran *et al*. 2001). Further, storage fungi, especially *A. niger* and *Penicillium* spp. are also able to grow at lower O_2 levels and in the presence of elevated CO_2 (Smith *et al.* 1986). Therefore, it may be that the grain used in this study contained more field fungi than storage fungi. Future research could focus on testing grain in the field prior to harvest, to confirm these results.

This study found that a few fungi were able to survive at the lowest storage temperature (20°C) and at high moisture content (15%) over six months. In contrast, Malaker *et al*. (2008) reported that storage fungi grow most quickly on moist grain at a temperature of about 30ºC, whereas at 12 to 15ºC, most storage fungi grow very slowly on grains with 15

to 16% moisture contents. However, Christensen and Kaufmann (1965) observed that *Penicillium* can spoil grain at low temperatures and at moisture content above 16%; they also reported that wheat was free of storage fungi for up to two years when stored at moisture content between 15 - 17% and at 20° to 25°C. The results of the present study and that of Christensen and Kaufmann (1965) indicate that fungi can grow slowly at low temperature and at ≥15% moisture content.

This study demonstrates the importance of testing grain for fungal contamination at frequent intervals, as fungi were isolated from apparently "healthy" grain. These fungi remain in a dormant condition, however if the grain is incubated a large part of fungi will become active, and the interactions with other microorganisms will start. Due to the importance of the contamination of wheat grain with potentially mycotoxigenic fungi and the effect of temperature and moisture content on the prevalence and growth of fungi, the extent of fungal contamination of wheat across Australia at optimal growth conditions during storage is explored in Chapter 3.

Chapter 3: Mycoflora in stored wheat grain vary between different states of Australia

Source: praytogodtoday.blogspot.com

3.1 Introduction

Many different fungi can infect wheat grains during or after harvest and during transfer of the grain into storage (Riba *et al*. 2008). Fungal contamination is a major concern because it can cause economic losses to stored grain as well as a potential decline in human and animal health if the contaminated grain is consumed (Marasas 1995). In Australia, there is limited recent information about the mycoflora associated with wheat grain and their distribution across the main production areas (Berghofer *et al*. 2003). A survey by Berghofer *et al*. (2003) determined the mycoflora associated with Australian wheat in flour milling fractions and end products, and the most common fungi found were *Aspergillus, Penicillium, Cladosporium* and *Eurotium* spp. An earlier survey by Blaney and Williams (1991) found that Australian-grown grains including maize, sorghum and wheat were contaminated with *Alternaria* and *Fusarium*. Other fungi previously reported on Australian grain are discussed in Chapter 1.

The objective of this study was to isolate and identify the species of postharvest fungi present in stored wheat grain across five states of Australia. These regions have a wide range of climatic conditions; rainfall decreases from north to south, with the Southern Region (Victoria and South Australia) having a temperate climate and the Western Region (Western Australia) a Mediterranean climate, whereas the Northern Region (New South Wales) has a climate ranging from temperate to subtropical depending on the growing area. Soils in the Southern and Western Regions are poor (low fertility) with many subsoil constraints, while the Northern Region has relatively good soils. The hypothesis of this study was that differences in storage fungi exist between the different states of Australia.

3.2 Material and Methods

3.2.1 Sample collection

A total of 23 newly harvested (February 2012/2013 and February 2013/2014) non-chemical treated Australian standard hard wheat samples were collected from five different states (QLD, VIC, SA, WA and NSW) in Australia (Fig 3.1). The samples locations that were chosen were geographically diverse to encompass most of the major wheat-growing areas.

The wheat samples (1 kg) were taken from farm bins (50-150 t capacity). The sampling ports were 1 and 2 m from the top of the grain surface, and the two lots of sample were

mixed and stored in a 2 kg metal sample can. All samples were sent to the laboratory, within 2 days of collection and stored at -20°C. Before using, the samples were kept at 5°C for 2 days to defrost. The defrosted wheat samples were moved to room temperature (25°C) for 24 hrs and then placed into a sealed chamber (2 L) and allowed to equilibrate at 25ºC and 55% relative humidity for one week. Moisture content (wet basis) was checked with an electronic moisture meter (Graintec HE 50 electronic moisture meter, Graintec Pty Ltd, Toowoomba, Australia). The wheat samples were adjusted to 15% moisture content by adding a calculated volume of distilled water into a sealed flask (3L) at 25ºC for 1 week.

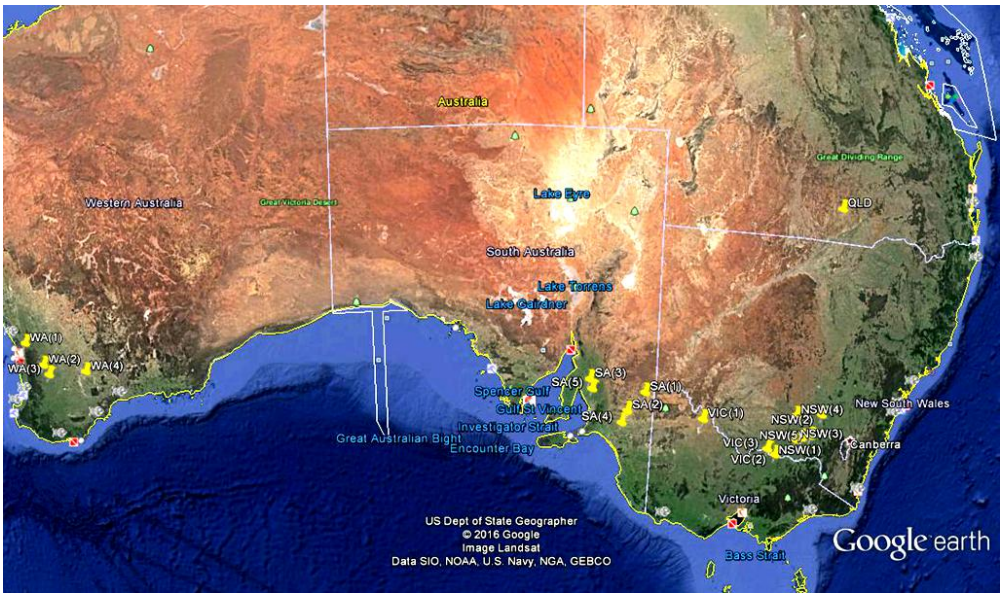


Figure 3.1 The collection locations of wheat grain from farms across different states of Australia: Western Australia (WA); South Australia (SA), New South Wales (NSW), Queensland (QLD) and Victoria (VIC).

3.2.2 Isolation of fungi from grain

Each farm sample was divided into four sub-samples to allow for four different isolation Methods. In Method 1, grains were surface sterilised by immersion for five min in 2.5% sodium hypochlorite followed by three washes in sterile distilled water, and dried on sterile filter paper for ten min in the laminar flow. Method 2 grains were not surface sterilised. All grain from these two treatments was plated onto full strength potato-dextrose-agar (PDA; Becton Dickinson and Company Sparks, MD USA) with streptomycin (133 mg/l) and incubated at room temperature (20-25ºC) in the dark. Methods 3 consisted of surface sterilised grain (as described above) that was placed in humidity chambers on filter paper

moistened with sterile distilled water. Method 4 grains were not surface sterilised and were placed in moist chambers. Methods 3 and 4 were also incubated at room temperature in the dark. From 24 hours to 14 days after plating for Methods 1 and 2, plugs of 5 mm^2 were taken from emerging colonies in each replicate, transferred onto fresh PDA plus streptomycin and incubated at 20-25ºC in the dark. Whilst for Methods 3 and 4, small fragments of mycelium growing from the grains in each replicate, were transferred by sterile needle onto fresh PDA plus streptomycin and incubated at 20-25ºC in the dark. There were three replicate plates of ten grains per plate for each of the four treatments.

A further sub-sample of grain from each of the 23 farms was also subjected to dissection. After the seed coat had been removed, the grain was dissected into six separate sections (Fig 3.2), to represent three external (s_1, s_2, s_3) and three internal sections (ss_1, ss_2, ss_3) according to Figure 3.2: (a) hairs of brush (s_1), (b) crease (s_2), (c) bracts (chaff, (s_3)), (d) radical (ss_1), (e) bran (ss_2) and (f) endosperm cells with starch granules (ss_3). A total of ten pieces for each section from each of the farm samples were plated onto PDA plus streptomycin, and incubated at room temperature in the dark. Plates were examined daily for the presence of fungal growth. Plugs of 5 mm^2 were taken from emerging colonies from 24 hours to 14 days, transferred onto fresh PDA plus streptomycin and incubated at 20-25ºC in the dark.

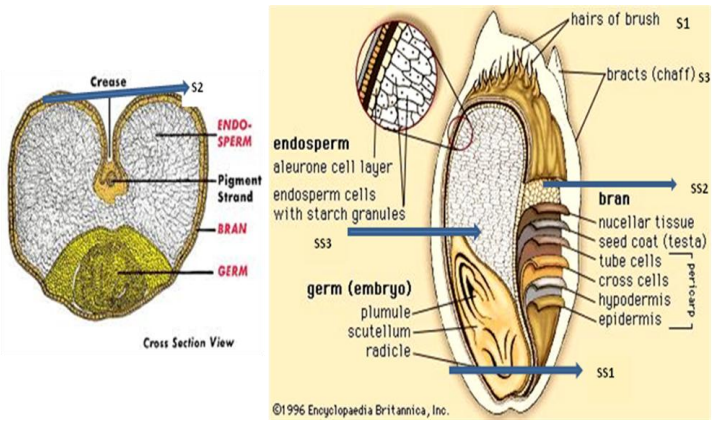


Figure 3.2 Sections of the wheat grain taken longitudinally (S1/S2/S3) and transversely (SS1/SS2/SS3). Source: Adapted from Wheat Foods Council, "a Kernel of Wheat" www.britannica.com.

3.2.3 Mycological analysis

Fungal colonies were grouped based on culture morphology and identified using the morphological criteria of Barnett and Hunter (1972), Booth (1971), Ellis (1971), and Nelson *et al*. (1983). Selected isolates from each group were chosen on the basis of morphological and cultural similarities for further identification using molecular methods (Table 3. 1) and all isolates were also lodged with the Department of Agriculture and Food Western Australia (DAFWA) herbarium.

3.2.4 DNA extraction, amplification and sequencing of fungal isolates

Mycelium from individual isolates (Table 3.1) was frozen in liquid nitrogen, and ground to a fine powder. Genomic DNA was extracted using a hexadecyl trimethyl ammonium bromide protocol but modified by the addition of 100 μg/ml Proteinase K and 100 μg/ml RNAse A to the extraction buffer as per Andjic *et al*. (2007). The internal transcribed spacer (ITS) region of the ribosomal DNA operon was amplified using the primers ITS-1F (Gardes and Bruns 1993) and ITS4 (White *et al*. 1990). PCR products were purified and sequenced as described by Sakalidis *et al*. (2011). Sequences were tentatively identified by using a BLAST search to match to known sequences in GenBank, and confirmed by phylogenetic analysis using Geneious.

Table 3.1 Isolates selected for ITS sequencing based on grouping according to morphological features. WAC number allocated by DAFWA Herbarium.

Species	WAC Numbers
Alternaria alternata	13842, 13843, 13844, 13845, 13846, 13847, 13848
Alternaria chartarum	13826, 13827
Alternaria infectoria	13849, 13850, 13851, 13852, 13853, 13854, 13855, 13856, 13857, 13858
Alternaria malorum	13818
Aureobasidium pullulans	13820, 13821
Aureobasidium sp.	13824, 13825
Cladosporium cladosporioides	13838, 13839, 13840, 13841
Cladosporium herbarum	13833
Eutiarosporella sp.	13806, 13805
Fusarium equiseti	13829
Fusarium oxysporum	13828
Fusarium pseudograminearum	13837
Fusarium tricinctum	13834, 13835
Mucor circinelloides	13817
Nigrospora oryzae	13836
Nigrospora sp.	13823
Penicillium cordubense	13831
Penicillium dipodomyicola	13832
Stemphylium sp.	13859

3.2.5 Diversity analysis of wheat samples

In addition to ITS analysis, a bulked sample consisting of up to10 g of grain was prepared by combining 2 g of grain from each farm (one bulk sample for each state). This was milled to a fine powder in a coffee grinder (Breville, Australia) and used for DNA extraction by the Australian Genome Research Facility (AGRF). The composition of species diversity in high-throughput amplicon sequencing data was carried out using the Quantitative Insights Into Microbial Ecology (QIIME) software package version 1.6 (http://qiime.org; Caporaso *et al*. 2010).

3.2.6 Statistics

Statistical analysis for the two methods on PDA and filter paper (with and without surface sterilisation) and for the six sections across different states provided the least reliable measure of density for the fungal isolations, the isolation relative density (RD) of each genus was calculated according to González *et al*. (1995), where RD (%) = (Number of isolates of a genus or species/Total number of fungi or genera isolated) × 100.

Differences in the fungal communities between states using both the number of different isolates in each state and the frequency with which they occurred were assessed using the freeware Paleontological statistics software package (PAST) V 3.01 (Hammer *et al*. 2001, available for download from http://folk.uio.no/ohmmer/past/). The multiple farms in each state constituted replicates for the analysis. The Non-Metric Multidimensional scaling (nMDS) statistical technique was used to visualise patterns of fungal diversity across states, with stress values of less than 0.2 being interpreted as providing a useful figure (Clarke and Warwick 2001). One-way non-parametric multivariate analysis of variance (PERMANOVA) was used for statistical comparisons of the fungal communities from each state. Following significant results in PERMANOVA, similarity percentages (SIMPER) were used to determine which isolates made the greatest contribution to differences between states (Hammer and Harper 2006). Farms within states could not be compared with the above approach because there was no replication within each farm. However, if each farm is regarded as an 'ecological community,' the ecological statistics species richness (the number of species from the farm), species diversity (Shannon diversity, $H' = -\Sigma pi$ (ln pi, where pi is the proportion of isolates from species i), and equitability ($E = H'/H'max$, where $H'max$ = the highest value H' can assume for the data for a farm) (see Krebs 1999). These statistics can be compared at a significance level of 0.05 using a

permutation test for equality (Hammer and Harper 2006, p. 197). Given the multiple tests, the significance value of 0.05 was adjusted for each state using the Bonferroni correction.

3.3 Results

Fungi associated with stored grain in Australia

From the 23 farm samples from across Australia a total of 498 isolates from 16 genera were isolated. These included *Alternaria* spp., *Aspergillus* sp., *Aureobasidium* sp., *Cladosporium* spp., *Drechslera* sp., *Fusarium* spp., *Mucor* sp., *Nigrospora* sp., *Penicillium* sp., *Rhizopus* sp., *Setosphaeria* sp., *Stemphylium* sp., *Eutiarosporella* spp., *Ulocladium* sp., *Epicoccum* sp., and an undescribed genus from the Hypocreales. The number of isolates per state were: Western Australian grain (231), SA (133), Victoria (67), NSW (51) and 16 isolates from Queensland (Table 3.2).

Identification of fungal species by screening assays

In the PDA treatments, the number of isolates retrieved was similar with and without surface sterilisation ($p = 0.34$) although far more individuals were retrieved without surface sterilisation ($p < 0.01$). Similar results were obtained for the moisture chamber treatments, where the number of isolates retrieved did not differ depending on surface sterilisation ($p = 0.54$), but the number of individuals retrieved was greater without surface sterilisation (($p < 0.01$) (Table 3.3). Interestingly, 139 isolates were obtained from grain sections (97 from external and 42 from internal sections), leading to statistically significant differences in both the number of isolates ($p = 0.041$) and the number of individuals retrieved ($p < 0.01$) between internal and external sections. When comparing isolation methods, similar numbers of isolates were retrieved using the PDA method when compared to the filter paper method ((272 isolates compared to 87 isolates, $p = 0.56$), although more individuals were retrieved using PDA ($p = 0.01$).

Looking at isolation of different genera, the highest numbers of fungi such as *Alternaria, Aspergillus, Rhizopus, Mucor and Nigrospora* were isolated from grain that was placed on PDA in comparison to the moisture chambers (Table 3.3). The section method was most suitable for the detection of *Aureobasidium pullulans* and *Penicillium dipodomyicola* as these species were the most frequent fungal species isolated from the excised sections of grain (Table 3.3). Other genera such as *Cladosporium* and *Fusarium* were isolated in equal numbers using all methods.

Table 3.2 Species and numbers of fungal isolates obtained from wheat samples isolated from WA (eight farms), SA (six farms), NSW (five farms), VIC (three farms) and one farm from QLD.

Species	**Farms sampled**																					
	WA 1	**WA 2**	**WA 3**	**WA 4**	**WA 5**	**WA 6**	**WA 7**	**WA 8**	**SA 1**	**SA 2**	**SA 3**	**SA 4**	**SA 5**	**SA 6**	**VIC 1**	**VIC 2**	**VIC 3**	**NSW 1**	**NSW 2**	**NSW 3**	**NSW 4**	**NSW 5**	**QLD**
Alternaria alternata	3	11	4	0	0	0	0	0	0	0	2	1	0	0	2	2	2	1	0	0	0	1	4
Alternaria chartarum	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0
Alternaria infectoria	1	8	2	0	40	36	48	32	0	25	28	19	6	16	9	16	10	1	0	0	0	0	4
Aspergillus spp.	0	1	0	1	2	0	0	0	5	0	0	0	0	0	0	0	1	0	1	0	0	0	0
Aureobasidium pullulans	3	3	2	0	0	0	0	0	1	1	1	0	0	0	1	3	1	2	0	7	5	0	5
Aureobasidium sp.	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cladosporium cladosporioides	0	2	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0	0	1	0	0
Cladosporium herbarum	0	2	1	0	0	0	0	0	0	1	1	0	0	0	0	0	3	0	0	0	0	0	0
Drechslera sp.	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Epicoccum nigrum	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Eutiarosporella sp.	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
Fusarium tricinctum	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fusarium equiseti	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Fusarium oxysporum	0	0	0	0	0	0	0	2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Fusarium pseudograminearum	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Mucor circinelloides	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0
Nigrospora spp.	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 3.2 (continued). Species and numbers of fungal isolates obtained from wheat samples isolated from WA (eight farms), SA (six farms), NSW (five farms), VIC (three farms) and one farm from QLD.

Species	**Farms sampled**																					
	WA 1	**WA 2**	**WA 3**	**WA 4**	**WA 5**	**WA 6**	**WA 7**	**WA 8**	**SA 1**	**SA 2**	**SA 3**	**SA 4**	**SA 5**	**SA 6**	**VIC 1**	**VIC 2**	**VIC 3**	**NSW 1**	**NSW 2**	**NSW 3**	**NSW 4**	**NSW 5**	**QLD**
Nigrospora oryzae	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Penicillium cordubense	0	0	0	0	0	0	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Penicillium dipodomyicola	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	20	0	0	0	0
Rhizopus spp.	1	0	1	8	0	0	0	0	4	1	0	1	5	5	2	4	3	2	0	1	0	4	0
Setosphaeria rostrata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Stemphylium spp.	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ulocladium sp.	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Unidentified species from the Order Hypocreales	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 3.3 Fungal isolates obtained from wheat samples isolated on potato dextrose agar (PDA), filter paper, or plating of external and internal sections.

	PDA		Filter paper		Grain Sections	
Species	**Sterilised**	**Non-surface sterilised**	**Sterilised**	**Non-surface sterilised**	**External sections**	**Internal sections**
Alternaria alternata	2	16	1	7	7	0
Alternaria infectoria	81	96	24	20	54	26
Alternaria chartarum	0	2	0	0	0	0
Aspergillus sp.	0	7	0	2	1	1
Aureobasidium sp.	0	0	0	0	0	1
Aureobasidium pullulans	1	5	1	4	18	6
Cladosporium herbarum	0	1	0	2	3	2
Cladosporium cladosporioides	0	4	0	1	3	0
Drechslera sp.	0	4	0	0	0	0
Epicoccum nigrum	0	1	0	0	0	0
Eutiarosporella sp.	2	0	0	1	0	0
Fusarium tricinctum	0	2	0	0	0	0
Fusarium oxysporum	0	0	0	3	0	0
Fusarium equiseti	0	1	0	0	0	0
Fusarium pseudograminearum	0	1	0	0	0	0
Unidentified Hypocreales	0	1	0	0	0	0
Mucor circinelloides	0	3	0	0	1	0
Nigrospora sp.	0	1	0	0	1	0
Nigrospora oryzae	0	4	0	1	0	0
Penicillium cordubense	2	2	0	2	0	0
Penicillium dipodomyicola	1	5	0	3	8	6
Rhizopus sp.	1	17	0	14	2	0
Setosphaeria rostrata	0	1	0	0	0	0
Stemphylium sp.	0	1	0	0	0	1
Ulocladium sp.	0	1	0	0	0	0
TOTAL	**91**	**180**	**26**	**60**	**98**	**43**

Distribution of fungal species across states

Visual inspection of the nMDS plot indicated that NSW had a distinct fungal community in the grain samples, whereas there was considerable overlap in the other states (Fig. 3.2). This was confirmed in PERMANOVA, where there was a significant (pseudo $F = 1.83$, $p = 0.04$) difference across all states. In paired comparisons between states, there were significant differences in the fungal communities between grain from NSW and SA ($p < 0.01$), NSW and Victoria ($p = 0.03$), and NSW and WA ($p = 0.04$). Considering all states together, SIMPER showed that the pattern of occurrence of *Alternaria infectoria* explained 48% of the variability across samples with a relative abundance of 33% in Western Australia, 18% in South Australia, 7% in Victoria. *Alternaria infectoria* was also the major contributor to each significant paired comparison between states, with the scope of its contribution varying from 42.0% between NSW and Victoria to 45.0% between NSW and SA. The next most common species was *Rhizopus* spp., which contributed 8.4% to the variability across states with 3% relative abundance in South Australia. Other fungal genera isolated had lower abundances. QLD was not included in this analysis as it only had one farm sample.

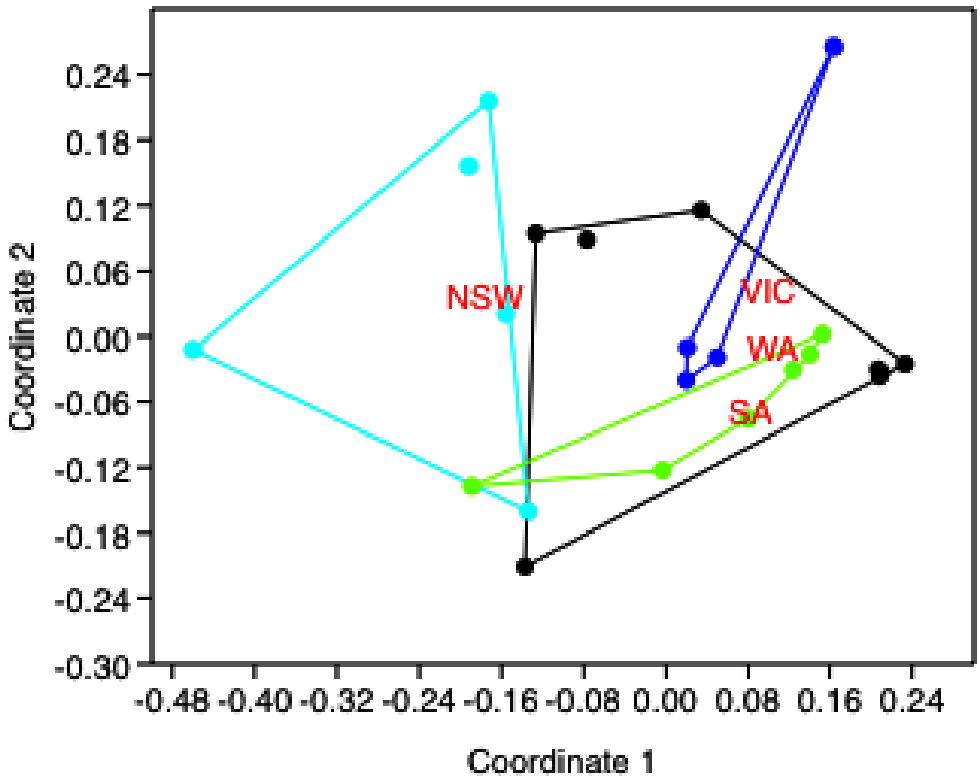


Figure 3.2 Non-metric multidimensional scaling model (nMDS) plot comparing the fungal communities isolated from grain samples collected from farms across Australia in each state: New South Wales (NSW), Victoria (VIC), Western Australia (WA), South Australia (SA). Minimum convex hulls (the smallest polygon embracing all points for a state) are shown. The stress was 0.13 (Clarke KR, Warwick RM (2001)).

Molecular identification and phylogenetic analysis

From all isolates collected, a total of 45 isolates were subjected to molecular analysis to confirm their identity. Based on sequence data and phylogenetic analysis, seven of the *Alternaria* isolates appeared to be most closely related to *A. alternata*, two were related to *A. chatarum*, one belonged to *A. malorum* and ten to the *A. infectoria* clade (Appendix 1). Four isolates were closely related to *Cladosporium cladosporioides* and one isolate to *Cladosporium herbarum* (Appendix 2). In addition, two isolates of *Fusarium* were closely related to *Fusarium tricintum* and one each to *Fusarium pseudograminearium*, *Fusarium equiseti* and *Fusarium oxysporum* (Appendix 3). Two isolates of *Aureobasidium* aligned to *Aureobasidium pullulans* and two isolates

appeared to be putative new *Aureobasidium* species (Appendix 4). The phylogenetic analyses for *Nigrospora* indicated one isolate was close to *Nigrospora oryzae* and another appeared to be an unidentified *Nigrospora* sp. (Appendix 5). One isolate each of *Penicillum cordubense* and *P. dipodomyicola* were confirmed, and one isolate appeared to be an undescribed *Stemphylium* sp. (Appendix 6 and 7).

Diversity analysis

Diversity profiling analysis of bulked farm samples resulted in the identification of additional species that were not identified by ITS analysis (Table 3.4). Further, a total of 56 species were identified by diversity profiling analysis, compared to 23 species using traditional methods and ITS analysis. Based on sequence reads, in Western Australia *Paecilomyces* was the dominant genus, with 78% of all reads, followed by *Cryptococcus macerans* at 9% (Table 3.4). A second sample of grain from three WA farms from the 2012/13 season (Farms 1-3 in Fig 3.1) was also subjected to a separate diversity analysis as a seasonal comparison. In this earlier season, *Alternaria metachromatica* was the dominant sequence at 68% (data not shown).

In South Australia, the most common species based on the number of sequence reads was *Dendryphiella arenaria*, however unknown fungi also made up 23% of the reads. *Cryptococcus* was recorded in almost 40% of the SA sequences, with *C. macerans, C. victoriae* and *C. oeirensis* all being present (Table 3.4). Victorian grain also had high numbers of sequence reads of *Cryptococcus macerans* (48%) and *C. victoriae* (34%) (Table 3.4). In NSW, *Aspergillus* species made up the highest number of reads at 42%, followed by unidentified species at 24% (Table 3.4). *Cryptococcus* species were all less than 1% in NSW.

Table 3.4 Common fungal species identified in wheat grain samples based on sequence reads from bulked farm samples. The highlighted species were also isolated by traditional methods.

Species	NSW	WA	SA	VIC
Aspergillus sp	42%	0%	0%	0%
Cryptococcus macerans	0%	9 %	19%	48%
Cryptococcus victoriae	0%	5.41%	13%	34%
Cryptococcus oeirensis	0%	4%	6%	0%
Dioszegia hungarica	0%	0%	1.45%	0%
Dendryphiella arenaria	1.31%	0%	26%	5%
Udeniomyces pyricola	2%	0%	0%	0%
Epicoccum nigrum	0%	1.26%	0%	0%
Eurotiales sp	17%	0%	0%	0%
Fusarium graminearum	0%	0%	1%	0%
Lewia sp	0%	1.20%	0%	0%
Paecilomyces sp	11%	78%	0%	0%
Sporobolomyces roseus	0%	0%	6%	1.11%
Udeniomyces puniceus	0%	0%	0%	3.20%
Unidentified	24%	4.52%	23.50%	6%
Others less than 1%	3.10%	4.04%	4.60%	3%

The species identified by diversity profiling were further investigated via the literature to establish two broad groups of fungi that were present on stored wheat grain across Australia - wheat pathogens and saprophytes/non-pathogens (Table 3.5). The most dominant saprophyte was *Paecilomyces* spp. as it represented 89% of all isolations across four states, followed by *Aspergillus* sp. as one of the most abundant fungi (43% of all sequence reads). *Dendryphiella* was present on grain at 34% and Eurotiales at 17%. Basidiomycetous yeasts, mainly *Cryptococcus macerans* and *Cryptococcus victoriae* were found in the present study, with a high number of reads at 71 and 57%, respectively. Other Basidiomycetous fungi *Sporobolomyces*, *Udeniomyces* and *Dioszegia* were present on the grain but only with small reads at 5%, 3%, and 1%, respectively. The wheat pathogen *Fusarium graminearum* was found with small reads on grain at 1% (Table 3.5).

Table 3.5 Common pathogens and saprophytes of wheat identified in bulked wheat grain samples from across Australia based on sequence reads.

Identity	Phylum	Sequence reads (%)	Disease in wheat
Paecilomyces sp.	Ascomycete	89%	Non pathogenic
Aspergillus sp.	Ascomycete	43%	Black head mould of wheat
Dendryphiella arenaria	Ascomycete	34%	Non pathogenic
Cryptococcus macerans	Basidiomycete	71%	Non pathogenic
Cryptococcus victoriae	Basidiomycete	57%	Non pathogenic
Eurotiales sp.	Ascomycete	17%	Black head mould of wheat
Cryptococcus oeirensis	Basidiomycete	7%	Non pathogenic
Sporobolomyces roseus	Basidiomycete	6%	Black head mould of wheat
Udeniomyces puniceus	Basidiomycete	4%	Non pathogenic
Dioszegia hungarica	Basidiomycete	1%	Non pathogenic
Gibberella zeae	Ascomycete	1%	Fusarium head blight

3.4 Discussion

The variation in the numberof fungi across states

This study provides an insight into fungi currently present in healthy grain stored in silos throughout the wheat growing regions of Australia. The observations of different patterns of occurrence in fungal communities between states is interesting. These results agree with the hypothesis of this study that differences in storage fungi exist between the different states of Australia.

The interstate variation in the numbers of fungi may be explained by differences in climatic conditions during the growing seasons of each region and the variation of the mycoflora at those locations. The warmer climates of Western Australia and South Australia most likely provide a suitable environment for the growth of fungi in comparison to New South Wales and Victoria. This is supported by Riba *et al*. (2008) who demonstrated that some fungi such as *Aspergillus niger* are widespread in warmer climates, both in field crops and stored foods. The reasonable explanation for the results in the current study is fungi can be influenced by various environmental factors such as temperature, rainfall frequency and geographic distribution or extreme events (i.e., floods and cyclones in Queensland and New South Wales during the seasons collected). It can be concluded that major storage fungi were *Alternaria* spp., in the storage of wheat grain under Western Australia conditions. These findings agree with Pitt and Hocking (2003) who reported *Alternaria* as the main problem in Australian wheat, and these included *Alternaria alternata* and *A. infectoria*.

Alternaria has a worldwide distribution, is ubiquitous and includes various pathogenic plant and saprophytic species that can spoil grain pre-harvest or postharvest, if conditions are suitable. The high number of *Alternaria* isolations in the current study is similar to Patriarca *et al*. (2007) and Kulik *et al*. (2014) who found that *Alternaria* spp. were common spoilage fungi of crops in the field and caused postharvest decomposition of various grains including wheat and rye in Poland. In Australia, *Alternaria*, especially *A. alternata* and *A. infectoria*, has been reported on wheat, sorghum and barley (Pitt and Hocking 2006; Webley *et al*. 1997; Blaney and Williams 1991).

Contamination of wheat by *Alternaria* species is a concern as the genus has the ability to produce mycotoxins (Patriarca *et al*. 2007). Pitt and Hocking (2003) found that *Alternaria* produced a range of toxins in wheat including tenuazonic acid, altenuene, alternariol and alternariol monomethyl ether. The results of the present study indicate that it is essential to further study the toxigenic ability of *Alternaria* spp. and the prevalence of *Alternaria* in stored wheat across Australia due to its dominant presence. However, there should be a particular focus on wheat that is at higher risk of mould development, for example when storage conditions are breached allowing ingress of moisture, the presence of grain storage insect pests, or when the growing season has been particularly wet. As *Alternaria* also has the ability to produce mycotoxins, it will be critical to monitor stored grain for the presence of these toxins in the future, should on-going monitoring for the pathogen show a continued high level presence.

Other species observed in high counts from almost all the wheat grain samples included *Aureobasidium*, *Rhizopus* spp., *Cladosporium* and *Penicillium*. These results concur with Berghofer *et al*. (2003, Table 1.1) who detected *Aureobasidium*, *Alternaria* and *Fusarium* in Australian wheat. Shipton and Chambers (1966; Table 1.1) found *Cladosporium*, *Rhizopus* spp., *Penicillium* spp., *Alternaria* spp., *Stemplium* spp., and *Ulocladium* spp., associated with Western Australia wheat. Other fungi previously reported on Australian grain are also discussed in Chapter 1 (Table 1.1). In a similar study by González *et al*. (1999), *Cladosporium* and *Rhizopus* species were isolated from wheat in Argentinia whereas Riba *et al*. (2008) found the dominant storage fungi in Algerian wheat were from the genus *Aspergillus*, mainly *A. flavus*, *A. niger* and *A. versicolor*. Mostafa *et al*. (2011) also isolated five different genera from wheat in Iran belonging to *Cladosporium* spp., *Alternaria* spp., *Penicillium* spp., *Aspergillus* spp. and *Fusarium* spp. as internal and external mycoflora. It seems that most microorganisms

are present on the grain surface and only a few species can live internally in cereal seeds, with more external than internal pathogens isolated in the present study.

Effect of samples size and isolation methods on recovery of fungi associated with stored grain in Australia

In the present study a higher number of fungi were isolated from grain on PDA plates compared to moisture chambers. Niaz and Dawar (2009) found the agar plate method is the most appropriate for the detection of *Aspergillus* spp., *Cladosporium* spp., *Curvularia* spp., and *Rhizopus* spp. on maize seeds. Mathur and Neergaard (1970) and Khan *et al*. (1988) also preferred the use of the agar plate method to the blotter method (moist chambers) for the isolation of *Curvularia* spp., and *Drechslera* spp. from rice seed. Adesemoye and Adedire (2005) used different cereals incorporated into the media including, corn meal dextrose agar, millet-meal dextrose agar and sorghum-meal dextrose agar. These media readily supported the growth of an extensive variety of mycoflora without any other additives (Adesemoye and Adedire 2005). Therefore, in future studies it might be beneficial to use substrates in the isolation media that are similar to the substrate being isolated from. For example, use wheat as a substrate incorporated into the agar medium when isolating from wheat grains.

It is important to find methods that increase the diversity of fungal species isolated on agar to levels similar to those found using molecular methods directly from the grain. Isolation on agar media provides information on viability of inoculum in the infected seed sample. However, Marcinkowska (2002) indicated that the blotter method (moisture chamber) is commonly used when regular seed health testing is carried out and that the agar test is non practicable as this test can cause variations in the growth of fungi depending on the species. The blotter method combines advantages of in *vitro* examination with *in vivo* observations. Staining was also recommended by Marcinkowska (2002) to detect seed-borne pathogens that are biotrophs or may grow on artificial substrate but very slowly, and generally are not able to develop fruiting structures. The diversity profiling analysis identified a number of fungi potentially present on the grain that were not isolated using traditional methods. The reasonable explanation for this finding is the grain used in the diversity sample was very much larger than the sample (30 grains for each of the 23 farms) assessed using traditional isolation methods, which may affect total grain surface area and so the relative likelihood of contamination with fungi.

Diversity analysis

This method may be useful for the detection and identification of pathogenic fungi without isolation of cultures for morphological determination. Of most concern was the presence of *Cryptococcus macerans* which has been described as a human pathogen and the cause of diseases such as Meningoencephalitis (Lindsberg *et al*. 1997). Additionally, *C. victoriae* was identified, although has previously only been reported from the Antarctic environment (Montes *et al*. 1999). *C. oeirensis* was detected in three states and is also known to occur in Denmark where it is referred to as a 'black head mould' or weak pathogen on ripening or ripe wheat heads (Nicolaisen *et al*. 2014). *Cryptococcus* species can spread by wind or human activities such as shipping and handling to silos. The data obtained in this study indicate that the human fungal pathogen *C. macerans* is present on grain at reasonably high levels and therefore is of epidemiological significance and importance. The occurrence of the other *Cryptococcus* species in stored grain is unusual for Australia as to our knowledge these species have not recorded on wheat before. Ghannoum *et al*. (1992) reported that synthetic amino acid medium-fungal and yeast nitrogen based media are the best to support the growth of yeasts such as *Cryptococcus* species, these media might be useful in future for postharvest grain storage isolations to confirm if *Cryptococcus* is present and viable.

A recent study of the mycobiome of Danish wheat, using a similar diversity analysis method, reported different saprophytes and pathogens including *Alternaria, Cladosporium, Phaeosphaeria, Fusarium* and *Microdochium* and also *Cryptococcus tephrensis* and *Cryptococcus victoriae* (Nicolaisen *et al.* (2014). The present study is different to their study because our focus was on the fungi that are present in storage rather than at harvest. However, both studies indicate through diversity profiling analyses that more fungi are present on grain than what we previously knew. Such microbiome studies could assist in better management practices in the future by taking whole fungal populations into consideration when designing postharvest facilities.

Other fungi detected using the diversity profiling technique were also unexpected. For example *Dendryphiella arenaria,* which is frequently found in marine environments, was observed in all four samples but was particularly high in the South Australian grain. Previously, *Dendryphiella arenaria* has only been isolated from plant debris in sea foam and from marine sediments (Michaelis *et al*. 1987). Similarly, sequences of *Sporobolomyces roseus* were also obtained from all samples but were highest in the South Australian grain. This species has also been found in seawater (Hernandez-

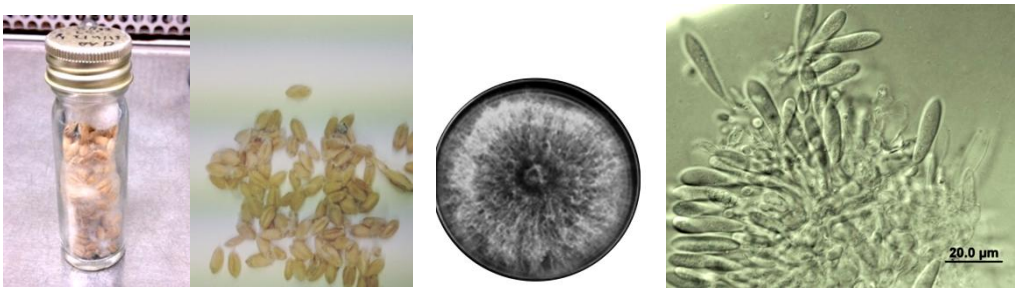
Saavedra *et al*. 1992; Gadanho *et al.* 2003) and freshwater (Libkind *et al.* 2003). An explanation for the presence of these fungi is that the spores were present in the atmosphere and were spread to the storage facilities when the grain was harvested and transported to the silo.

Typical soil fungi such as *Aspergillus* spp. were also found on wheat, indicating that this group of fungi can colonise wheat pre- or immediately postharvest, which has been demonstrated to occur if the grain has moisture content in equilibrium with a relative humidity between 70 and 90 % (Flannigan 1978). Isolates belonging to the genus *Paecilomyces* present on Western Australian grain have been isolated from *Taxus mairei* and *Torreya grandis* from south east China and are known to produce a wide range of bioactivity metabolites (Wang *et al*. 2002). Why such a high percentage of *Paecilomyces* spp., were detected in WA grain samples is not known. However, *Paecilomyces* are common environmental moulds (Domsch *et al.* 1980) and can grow under warm and dry conditions (Ying and Feng 2004), which are conditions typical to WA.

Conclusion

In conclusion, this study found that the mycoflora of stored wheat varies between Australian grain growing regions, and that different species of fungi are present on the grain surface when compared to the internal portion of the grain. This study revealed that *Alternaria* occurs with a high frequency in comparison to other genera, and that some pathogens of high risk to humans, either directly (e.g. *Cryptococcus*) or indirectly (e.g. *Fusarium* which produces mycotoxins) may also be present. The presence of fungi with the abillity to produce toxins could pose chronic adverse health effects to human and livestock fed on contaminated wheat or wheat products. Future investigations should include the toxigenic potential of Australian isolates of *Alternaria* and *Fusarium* species considering the frequency at which they were isolated from across Australia. Further work should also be conducted on the potential presence of *Cryptococcus* species, using both traditional and advanced technologies to determine if the genus is a common occurrence on stored grain or not. If common, it would be useful to determine how it is entering grain silos and under what conditions it will pose a threat to human and animal health.

Chapter 4: First report of *Eutiarosporella* species on stored wheat



4.1 Introduction

In 2012, wheat samples showing visual symptoms of fungal infestation were obtained from Cooperative Bulk Handling. The grain was cultured on agar and in moisture chambers and two species were putatively identified as *Eutiarosporella* based morphological and molecular analysis (Chapter 3). Subsequently, additional isolates were collected from stored wheat grain from farms in Western Australia during 2013 and 2014.

The genus *Eutiarosporella* belongs to the family Botryosphaeriaceae, a large and cosmopolitan family that is morphologically diverse and comprises saprophytes or endophytes mainly of woody plants, and important plant pathogens of economically and environmentally important plants such as *Syzygium cordatum* (Myrtaceae), *Pterocarpus angolensis* (Leguminosae), *Terminalia catappa* (Combretaceae), *Acacia* spp. (Fabaceae) especially *A. melifera*, and woody species of *Leucadendron*, *Leucospermum* and *Protea* (Jami *et al*. 2012). The Botryosphaeriaceae are known to cause cankers and dieback on a range of woody plants worldwide (Sakalidis *et al*. 2011; Jami *et al*. 2012; Jami *et al*. 2013). Because of their frequent association with plant diseases there has been considerable interest in the systematics of species and genera in this family (Phillips *et al*. 2013).

The genus *Tiarosporella* was introduced by Höhnel (1919) and is considered a sexual genus in the Botryosphaeriaceae (Jami *et. al* 2012; Phillips *et al*. 2013; Slippers *et al*. 2013). More recently, Crous *et al*. 2015 further resolved the genera in the Botryosphaeriaceae and this included formally accepting *Tiarosporella* as *Eutiarosporella* in this large family. *Eutiarosporella* has been reported as a pathogen of grasses (Jami *et. al* 2012), conifers (Jami *et. al* 2012; Thambugala *et al*. 2014) and of the members of the Asteraceae and Zygophyllaceae (Thambugala *et al*. 2014). Thynne *et al*. (2015) also recently reported *Eutiarosporella* from wheat in Australia. Jami *et al*. (2012) demonstrated that *Tiarosporella* species have distinct cultural characteristics and conidial morphology as compared to other genera of the Botryosphaeriaceae. The present study details two species of *Eutiarosporella* associated with stored wheat grain, one of which is described as a new species (*Eutiarosporella pseudotritici-australis* sp. nov.).

4.2 Materials and Methods

4.2.1 Isolation of fungi from grain

Australian hard white wheat (*Triticum aestivum*) was sampled from Cooperative Bulk Handling (CBH) in 2012 and from farms in 2013 or 2014 (Chapter 3 and Table 4.1). Mycological observations and radial growth was measured using the CBH isolates and molecular work included both CBH and farm isolates. Isolation methods were described in Section 3.2.2.

Table 4.1 *Eutiarosporella* isolates used in this study.

Code	WAC Number	Species name	Source of isolate
BR4	WAC13805	*Eutiarosporella pseudotritici-australis* sp. nov	Farm
EB8	WAC13806	*Eutiarosporella pseudotritici-australis* sp. nov	Farm
MUEB	WAC13807	*Eutiarosporella pseudotritici-australis* sp. nov	CBH
EB6	WAC13808	*Eutiarosporella pseudotritici-australis* sp. nov	CBH
MUE4D	WAC13809	*Eutiarosporella dactylis*	CBH
MUE4A	WAC13810	*Eutiarosporella dactylis*	CBH
MUE4C	WAC13811	*Eutiarosporella dactylis*	CBH

MUE, BR, EB refer to cultures of the author; WAC refers to the Department of Agriculture and Food Western Australia culture collection isolate numbers.

4.2.2 Identification based on spore morphology and culture characteristics

To induce sporulation 14 g of gamma-irradiated grain (18.7% moisture content) was placed in McCartney bottles and inoculated with one of five pure cultures, including two isolates of *Eutiarosporella pseudotritici-australis* from CBH (WAC13807, WAC13808) and three isolates of *E. dactylis* from farms (WAC13809, WAC13810 and WAC13811). Cultures were exposed to each of three different light treatments to induce sporulation: in the first, cultures were incubated at 20ºC under UV light; in the second, cultures were incubated at 25ºC in normal light; and in the third cultures were incubated at 25ºC in the dark. The incubation time was three months for all treatments. There were three replicate McCartney bottles for each isolate in each light /temperature treatment. The grain was observed weekly for the development of pycnidia and/or perithecia. The spore structures of *Eutiarosporella pseudotritici-australis* sp. nov (WAC13807) and *Eutiarosporella dactylis* (WAC13810) from the third treatment were plated on PDA (Difco) with streptomycin (133 mg/l) or placed into a moist chamber and incubated at

room temperature (20-25ºC) in the dark. A small part of these cultures 14-22 d old was also transferred by a sterile needle onto glass slides to microscopically examine the morphology of the conidia and conidiophores at 100, 400 and 1000 x magnification. Dimensions of fifty random conidia and conidiophores of each of the isolate were measured at 400 x magnification as described by Sutton and Marasas (1976).

4.2.3 Radial growth rates of isolates on different media

Pure cultures of the same five isolates used in section 4.2.2 were grown at 25°C in the dark for one week on full-strength PDA. Plugs (5 mm diam.) were then cut from the edge of actively growing cultures and placed in the centre of Petri dishes (9 cm) containing one of three different media: 2% malt extract agar (MEA; 20 g of Becton malt extract and 20 g of Becton bacteriological agar in 1 L of distilled water); corn meal agar (CMA; 17 g of Becton CMA in 1 L of distilled water); and PDA. Plates were incubated for six days at one of seven different temperatures (4˚C, 10˚C, 15˚C, 20˚C, 25˚C, 32.5˚C and 37.5˚C). There were three replicate plates per temperature per agar medium to give a total of 315 plates. The growth of the isolates was evaluated by the colony size method (Adesemoye and Adedire 2005). Briefly, the radius of growth was measured daily from 2 to 6 days at right angles to each other. The mean rates of growth of the three replicates and the standard errors were then calculated. The experiment was repeated twice but as there were no significant differences between the two experiments only experiment two data are provided here. For isolates that did not grow at 37.5°C, plates were moved to 25°C to determine if the isolates were dead or alive.

The growth of the isolates at different temperatures on each medium was evaluated using repeated measures ANOVA, with factors of isolate and temperature and a repeated measure factor of day (days 2 – 6). There were five levels of the repeated measures factor to correct for any possible violation of the sphericity assumption of the degrees of freedom for the main effect of days, and all interactions involving it were corrected using the relevant Greenhouse-Geisser epsilon and an adjusted p was assessed to determine statistical significance ($P < 0.001$).

In addition to measuring growth on the above three media, five isolates (WAC13807, WAC13808, WAC13809, WAC13810 and WAC13811) were selected for additional studies on three different grain media. These were wheat dextrose agar medium (WDA), barley dextrose agar medium (BDA) and a canola dextrose agar medium (CDA), according to the methods of Adesemoye and Adedire (2005) with minor modifications. Briefly, 6 g of each of the three grains (whole wheat, barley or canola) was added to a

flat bottom flask; 160 ml of clean water was added and heated in a water bath for 1 h. This was then filtered through filter paper (Whatman International Ltd. Maidstone, England 150mm × 100 circles. no.1) and 6 g of dextrose and 4 g of agar were added to the filtrate. The volume of the mixture was made to 200 ml with distilled water and heated on a hot plate with steady stirring until the solution boiled. The resultant suspension was sterilised in an autoclave for 15 min at 121ºC. Plugs (5 mm diam.) were cut from the edge of actively growing cultures on PDA of each isolate and placed in the centre of Petri dishes (9 cm) containing one of the three different media. They were then incubated at 25ºC in the dark for 7 days. There were 3 replicate plates per medium and the experiment was repeated twice. Colony measurements were conducted as described above.

4.2.4 Phylogenetic analysis

Three isolates of *Eutiarosporella dactylis* (WAC13809, WAC13810 and WAC13811) and four isolates of *E. pseudotritici-australis* sp. nov (WAC 13805, WAC 13806, WAC 13807 and WAC 13808) were cultured on PDA as described above for one week. DNA extraction and sequencing was conducted as described in Chapter 3 (Section 3.2.4). Sequences were matched to known sequences using a BLAST search, and based on the results were then aligned to the dataset for the Botryosphaeriales of Crous *et al.* (2015) using Geneious. Additional *Eutiarosporella* and *Tiarosporella* sequences were also included (Thynne *et al.* 2015; Thambugala *et al*. 2014, Table 4.2).

Table 4.2 GenBank accession numbers of *Eutiarosporella* and *Tiarosporella* species used in this study.

Species	Country	GenBank accession No.	Refrences
Eutiarosporella tritici australis	Australia	KP309788	Thynne *et al.* (2015)
Eutiarosporella tritici australis	Australia	KP309787	Thynne *et al*. (2015)
Eutiarosporella tritici australis	Australia	KP309790	Thynne *et al*. (2015)
Eutiarosporella tritici australis	Australia	KP309791	Thynne *et al*. (2015)
Euiarosporella darliae	Australia	KP309786	Thynne *et al*. (2015)
Euiarosporella darliae	Australia	KP309792	Thynne *et al*. (2015)
Euiarosporella darliae	Australia	KP309793	Thynne *et al*. (2015)
Euiarosporella darliae	Australia	KP309795	Thynne *et al*. (2015)
Euiarosporella darliae	Australia	KP309789	Thynne *et al*. (2015)
Eutiarosporella pseudodarliae	Australia	KP309796	Thynne *et al*. (2015)
Eutiarosporella pseudodarliae	Australia	KP309794	Thynne *et al*. (2015)
Tiarosporella dactylidis	Thailand	KM978949	Thambugala *et al*. (2014)
Tiarosporella dactylidis	Thailand	KM978948	Thambugala *et al*. (2014)

4.3 Results

Based on spore morphology, culture characteristics, sequence data and phylogenetic analysis, a new species of *Eutiarosporella* was identified which we named *Eutiarosporella pseudotritici-australis* sp. nov. as it seemed to be a sub-clade of *E. tritici-australis* which was recently reported by Thynne *et al.* (2015). A second species was also identified and this appears to be identical to *Eutiarosporella dactylis* as described by Thambugala *et al*. (2014).

4.3.1 Morphological comparisons

Eutiarosporella pseudotritici-australis sp. nov. was observed to produce black perithecia on the grain and microscopic examination showed the presence of asci. These were isolated into pure culture, and perithecia were not observed again, only the anamorph stages were observed.

Examination of the isolates of the two *Eutiarosporella* species found that the conidia and conidiophore dimensions of *E. pseudotritici-australis* sp. nov. and *E. dactylis* differ in length, width, colour, shape and texture from other known *Eutiarosporella* species.

Isolates of *Eutiarosporella pseudotritici-australis* sp. nov. had solitary and straight conidia with a pitted outer surface and a basal appendage (Fig 4.1). Conidia of *E. pseudotritici-australis* sp. nov. were smaller compared to the conidia of *Eutiarosporella*

tritici-australis sp. nov and *E. paludosa* but larger than those of *Eutiarosporella pseudodarliae* and *E. dactylis* (Table 4.2).

Colony characteristics of *E. pseudotritici-australis* sp. nov. and *E. dactylis* were also different when compared to reports of other *Eutiarosporella* isolates *in vitro* (Table 4.2).

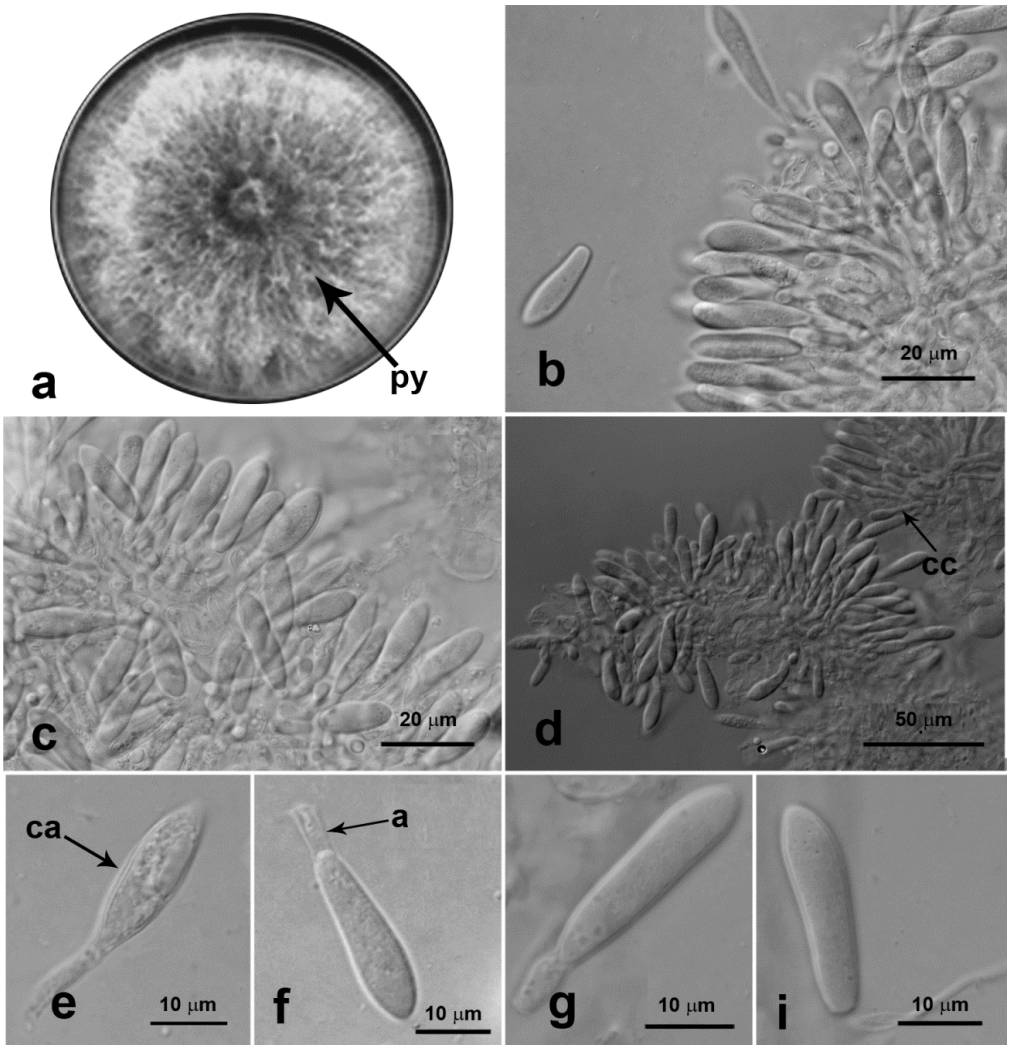


Figure 4.1 Macro and micro-structures of *Eutiarosporella pseudotritici-australis* sp. nov. (WAC13807): a) culture on PDA with pycnidia (py); b-d) conidiogenous cells (cc) producing conidia (ca); e-i) the appendage on conidia (a). Scale (b, c) = 20 µm, (d) = 50 µm, (e-i) = 10 µm.

Table 4.3 Morphological features of conidia and conidiophores and colony characteristics of *Eutiarosporella* species recorded in published literature and in the present study. Numbers in brackets refer to standard errors of the mean. (*) Standard error not reported. (-) Texture not included.

Morphological features	***Eutiarosporella dactylis* (Thambugala *et al*. 2014)**	***Eutiarosporella dactylis* (present study)**	***Eutiarosporella pseudodarliae* (Thynne *et al.* 2015)**	***Eutiarosporella tritici-australis* (Thynne *et al.* 2015)**	***Eutiarosporella paludosa* (Hyde 1993)**	***Eutiarosporella pseudotritici-australis* sp. nov (present study)**
Conidia dimensions (μm)	2.7 × 1.4 *	22.9 (± 0.77) × 6.7 (± 0.21)	12.39 (± 0.47) × 35.84 (± 0.60)	37.8 (± 0.65) × (12.25 (± 0.37)	37- 45 x 5.5-7*	20.8 (± 0.77) × 5.5 (± 0.16)
Appendage	Not observed	Not observed	Observed	Observed	Observed	Observed
Conidiogenous cell dimensions (μm)	7.8 × 1.8	7.9 × 3.6	7-16 × 3-7	7–12 × 3–5	Up to 14 long	8.7 × 2.9
Texture of conidia	Smooth	Rough coat	-	-	Smooth	Pitted coat
Shape of conidia	Ovoid, straight, oval	Clavate, ovoid to fusoid	Clavate to fusiform	Clavate to fusiform	Straight or curved	Solitary and straight
Colony characteristics on PDA	Flattened, fluffy, fairly dense, aerial, smooth surface greyish brown becoming black.	Woolly to cottony becoming grey olivaceous and darkening with age.	Mycelia darken to grey-white, olivaceous-grey/black, and light brown.	Initially white, discolour with age to white-grey, dark grey and olivaceous grey/black.	White, plumose producing immersed and superficial conidiomata	Woolly to cottony becoming grey olivaceous and darkening with age.
Colony growth on PDA	Colony covering 90 mm diam at 25ºC after 4 d.	Colony covering 90 mm diam at 20ºC. after 7 d.	Radial growth is rapid at 23ºC with 55–60 mm of growth, 2 days post inoculation and the 90mm plate is covered by 3 d.	Radial growth is rapid at 23ºC with 55–59 mm growth over 2 days (90mm plate)	Colony covering 90 mm at 22 ºC in 7 days.	Colony covering 90 mm plate at 20ºC after 7 d.

***Eutiarosporella pseudotritici-australis* sp. nov morphology and cultural characteristics**

On PDA the colonies of *E. pseudotritici-australis* sp. nov. (WAC13807, WAC13808) had limited aerial mycelium with woolly to cottony growth towards the margin of the Petri plate, and covered the 90 mm plate within five to six days. The growth was white at first, becoming grey to olivaceous and darkening with age with a blackish pigment diffusing into the medium, most noticeably after 14 days growth in the dark (Fig 4.1). On MEA *E. pseudotritici-australis* sp. nov. produced woolly cottony growth patterns (Fig 4.1). On CMA the mycelium was faded and sparse (Fig 4.2).

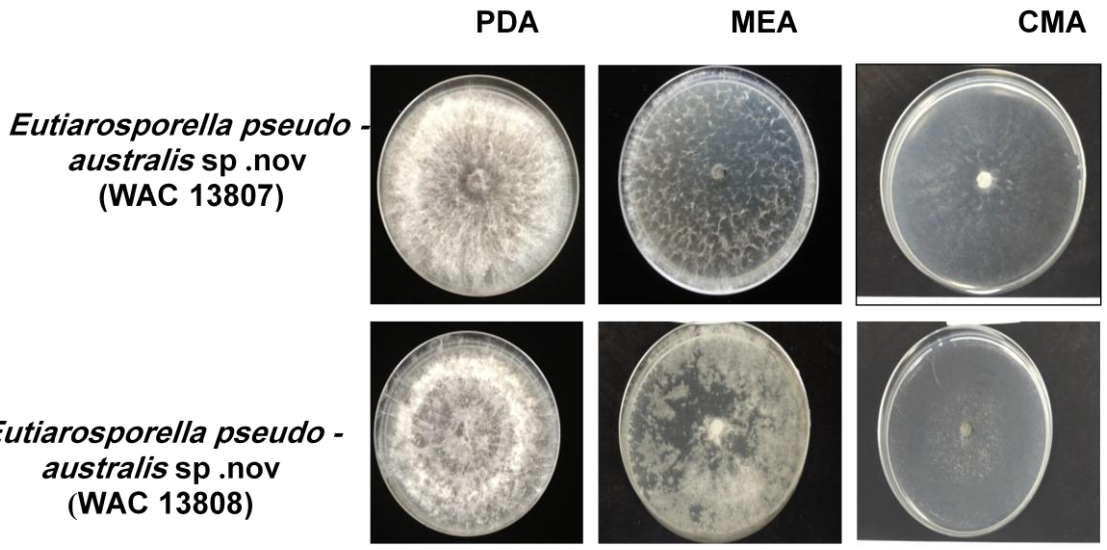


Figure 4.2 Colony morphology of isolates WAC13807 (top) and WAC13808 (bottom) of *Eutiarosporella pseudotritici-australis* sp. nov. after 7 days growth at 20°C on potato dextrose agar (PDA), malt extract agar (MEA) or corn meal agar (CMA).

Eutiarosporella pseudotritici-australis produced conidiogenous cells and conidia (Fig 4.1) in moisture chambers and on PDA within 14–21 days of incubation. Conidia averaged (20.8 × 5.5 µm), were pitted having small shallow craters on the surface, were solitary, thin-walled, and straight and the apex was pointed; and there was frequently a basal appendage attached (Fig 4.1 f, i). Fifty conidiogenous cells averaged (8.7 × 2.9 µm), and formed from the cells lining the inner walls of the pycnidia, and were holoblastic,

determinate, simple, cylindrical and slightly tapered towards the apex, and hyaline (Fig 4.1).

***Eutiarosporella dactylis* spore morphology and culture characteristics**

The colony growth patterns of the three isolates of *Eutiarosporella dactylis* (WAC13809; WAC13810; WAC13811) are shown in Figure 4.3. On PDA the colonies covered the Petri plates in four to five days, with sparse aerial mycelium. The colonies appeared woolly to cottony towards the margins of the Petri plates with some white branches of mycelium on the surfaces. These were white at first, becoming grey olivaceous and darkening in age. The mycelium was opaque with light coloured and sparse mycelium on CMA. The colonies of WAC13809 and WAC13811 on MEA were similar whilst WAC13810 had a stellate appearance (Fig 4.3).

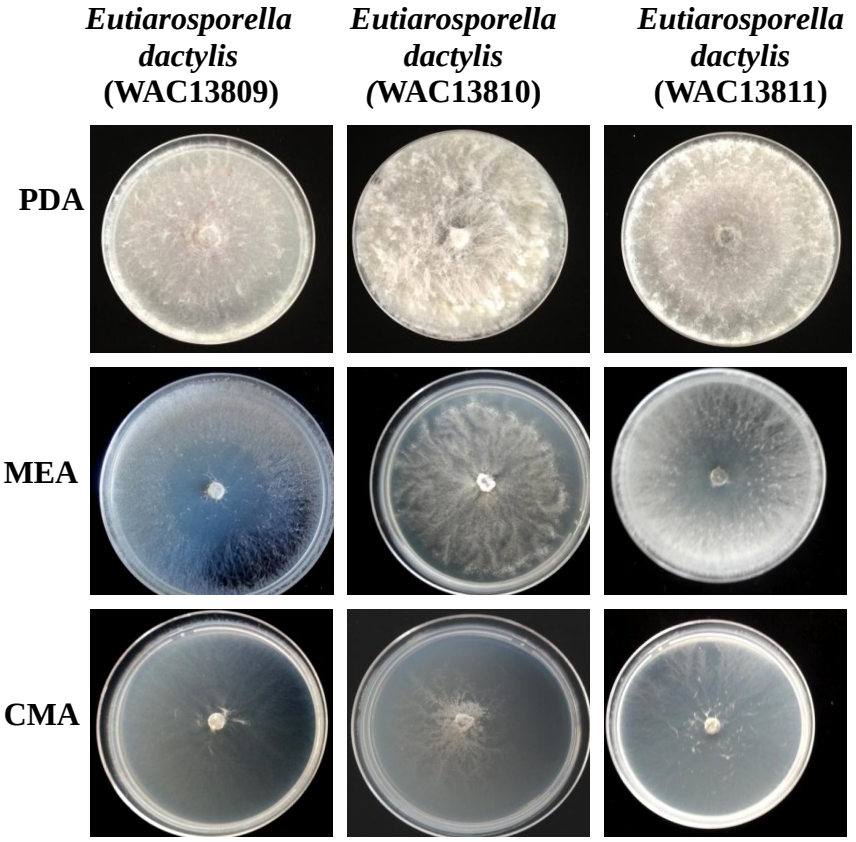


Figure 4.3 Colony morphology of *Eutiarosporella dactylis* isolates (WAC13809 WAC13810, WAC13811) after 7 days growth at 20˚C on potato dextrose agar (PDA), malt extract agar (MEA) and corn meal agar (CMA).

Conidiogenous cells of *Eutiarosporella dactylis* were cylindrical, hyaline, unbranched and averaged 7.9 x 3.6 μm. They formed from the cells lining the inner walls of the

conidiomata, which were phialidic, fusiform, holoblastic, determinate, cylindrical and hyaline. Fifty conidia averaged 22.9 × 6.7 μm, and were solitary, hyaline, rough coat, clavate, thin-walled, straight, ovoid to fusoid, apex obtuse, with a truncate base (Fig. 4.4).

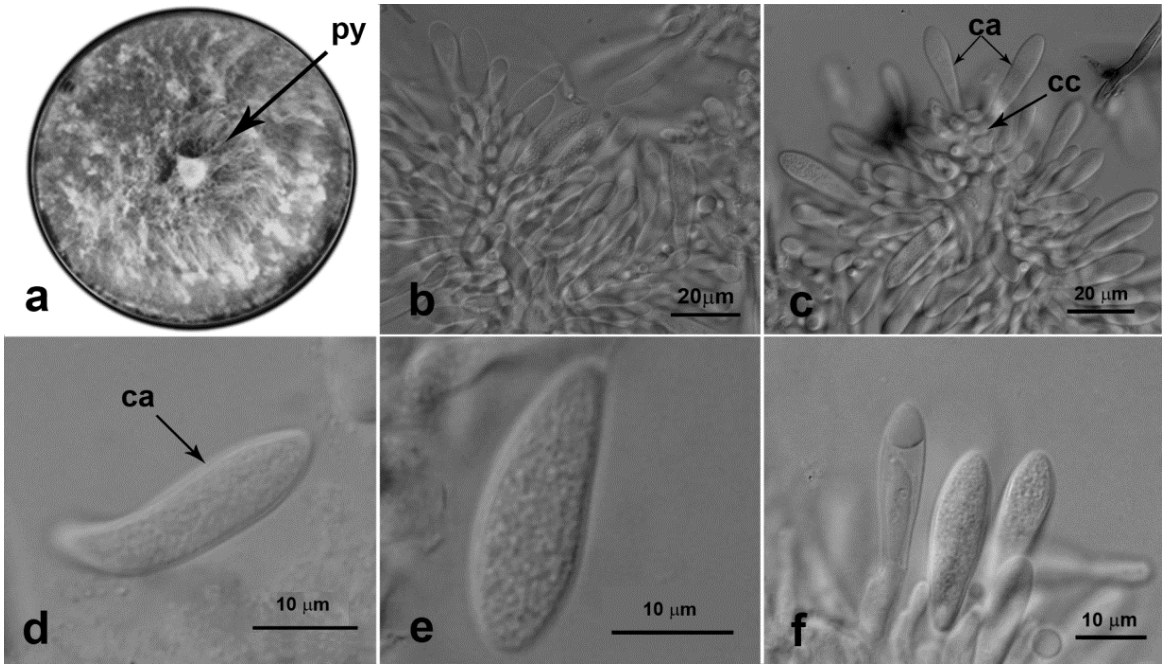


Figure 4.4 *Eutiarosporella dactylis* (WAC13810): a) culture on PDA with pycnidia (py); b, c) conidiogenous cells (cc) producing conidia (ca); d-f) conidia. Scale bar (b, c) = 20µm, (d, e, f) = 10 µm.

4.3.2 Radial Growth

On each medium, the response of the isolates over time varied with temperature as illustrated by significant three-way interactions between isolate, temperature and day (Fig 4.5). On PDA there were significant ($F_{80,400}$ = 12.71, P = 0.00) differences in interactions between the three isolates of *Eutiarosporella dactylis* EB32 (WAC13810), EB38 (WAC13811), and EB39 (WAC13809) and two isolates of *Eutiarosporella pseudotritici-australis* sp. nov. EB34 (WAC13808), and EB37 (WAC13807).

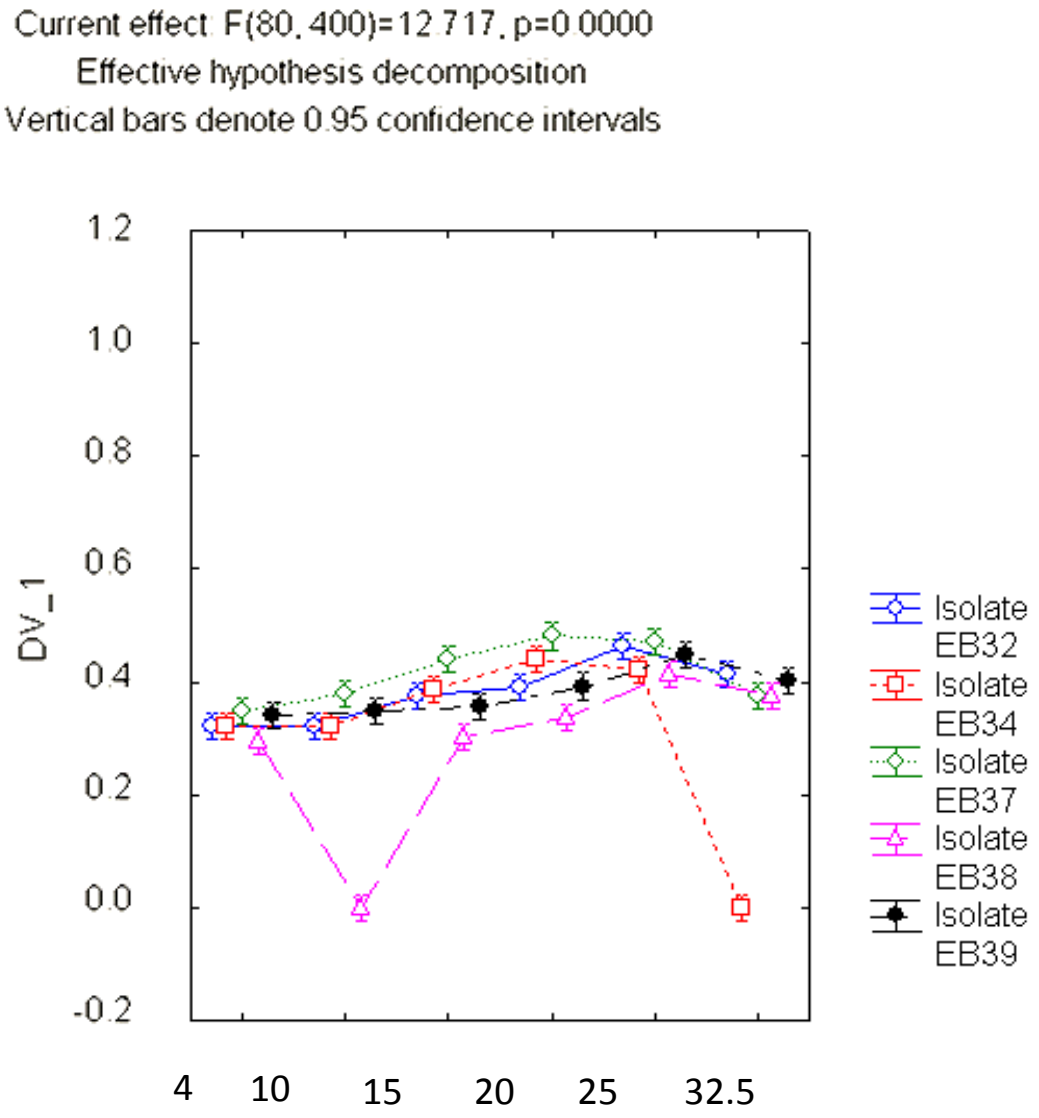


Figure 4.5 The radial growth of the three isolates of *Eutiarosporella dactylis* EB32 (WAC13810), EB38 (WAC13811), and EB39 (WAC13809) and two isolates of *Eutiarosporella pseudotritici-australis* sp. nov. EB34 (WAC13808), and EB37 (WAC13807) grown on potato dextrose agar varied with temperature as illustrated by significant ($F_{80,400}$ =12.72, p=0.00) three-way interactions between isolate, temperature and day. Vertical bars represent 95% confidence interval.

***Eutiarosporella pseudotritici-australis* sp. nov.**

There was a significant ($F_{24,70}$ = 25.4, $p < 0.01$) difference between the growth of isolates dependent on the interaction between isolates and temperature. The growth of WAC13808

was slower than WAC13807 on PDA, MEA and CMA (Fig 4.6). The maximum growth temperature on PDA for WAC13807 and WAC13808 was between 15- 25°C (Fig 4.6a). WAC13808 was unable to grow at 37.5°C, but WAC13807 grew very slowly.

On MEA the radial growth for the two isolates was slower than the radial growth on PDA. The optimum temperature for the two isolates was 25°C. The growth rate of WAC13807 was higher (15mm/d) than the growth of WAC13807 (10 mm/d) (Fig 4.6b).

On CMA there was a significant ($F_{24,70} = 12.3$, $p < 0.01$) effect of temperature on the growth of the two isolates compared to that on PDA and MEA (Fig 4.6c). The optimum growth was at 25°C for the both isolates.

On all media, both isolates continued to grow when returned to 25°C after their growth had stopped at 37.5°C.

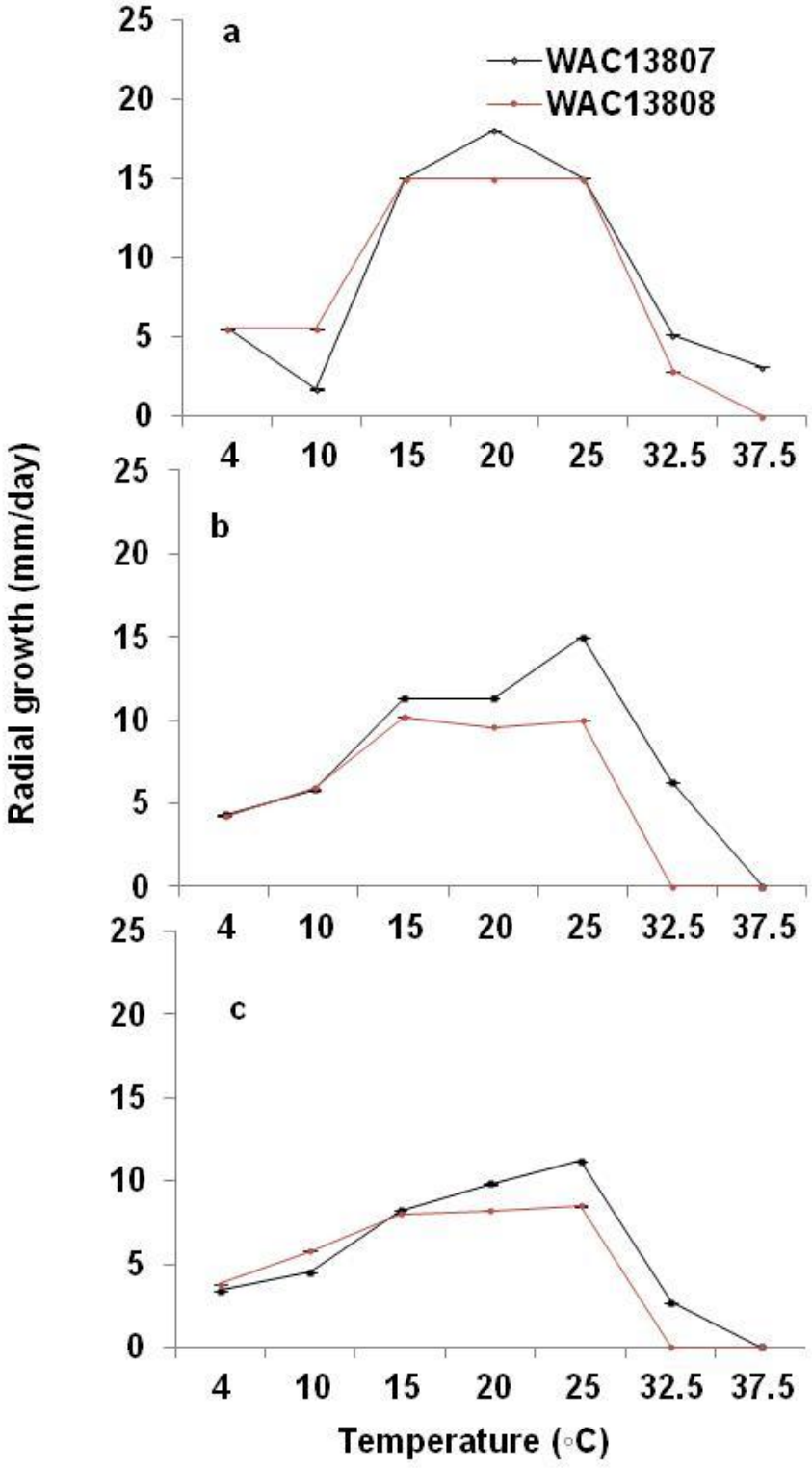


Figure 4.6 Mean radial growth rates (mm/day) of two isolates of *Eutiarosporella pseudotritici-australis* sp. nov. (WAC13807, WAC13808) on potato dextrose agar (a), malt extract agar (b), and corn meal agar (c) at different temperatures. Bars indicate standard errors of the mean.

Eutiarosporella dactylis

The maximum growth temperature for all isolates on PDA was 25°C (Fig 4.7a). All isolates were unable to grow at 37.5°C, but when the plates were moved to 25°C, growth was observed after 2 days. The growth rate for all isolates was higher on PDA than on MEA and CMA.

On MEA, there was a significant ($F_{96,280}$ = 18.2, P <0.001) difference in interactions between isolate, temperature and time elapsed. The radial growth rates of WAC13809 and WAC13810 were similar and grew quickest (18mm/d) compared to WAC13811, which was the slowest (15mm/d). The optimum temperature for WAC13809 was at 25°C and WAC13810 at 20°C (Fig 4.7b).

There was a significant ($F_{96,280}$ = 18.2, P <0.001) difference in growth between the three isolates on CMA, and the growth of the three isolates was reduced compared to the radial growth on PDA and MEA (Fig 4.7c). WAC13809 and WAC13810 grew the fastest (15mm/d) at 20°C and 25°C, respectively, whilst WAC13811 the slowest (9.3 mm/d) at 25°C.

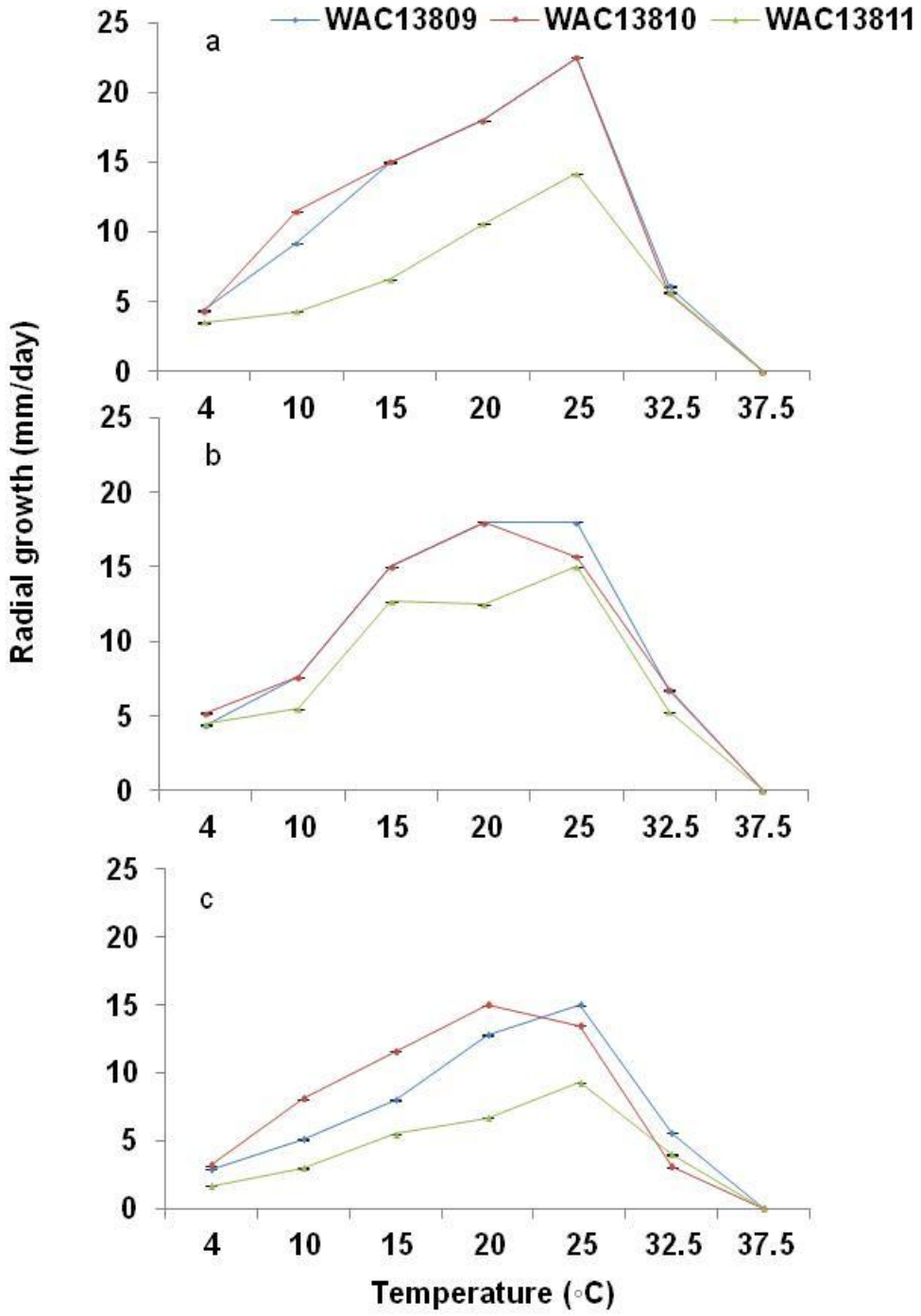


Figure 4.7 Mean radial growth (mm/d +/- SE) rates of the three isolates of *Eutiarosporella dactylis* (WAC13809, WAC13810, and WAC13811) on potato dextrose agar (a), malt extract agar (b), and corn meal agar (c) at different temperatures. Bars indicate standard errors of the mean.

Growth on grain media

The maximum growth of all isolates occurred at 25°C on all three media and all continued to grow at 32.5°C.

On wheat dextrose agar, the growth rate of *E. dactylis* isolate WAC13810 was higher than isolates WAC13809 and WAC13811. The growth of *E. pseudotritici-australis* sp. nov (WAC13807) was similar to the *E. dactylis* isolates (WAC13809 and WAC13811; Fig 4.8a).

The growth of *E. pseudotritici-australis* sp. nov was affected on barley dextrose agar medium and the resulting the growth rate was slower compared to the *E. dactylis* isolates (Fig 4.8b).

On the canola-based medium, the isolate of *E. pseudotritici-australis* sp. nov. (WAC13807) responded similarly to the three isolates of *E. dactylis* (WAC13809, WAC13810 and WAC13811).

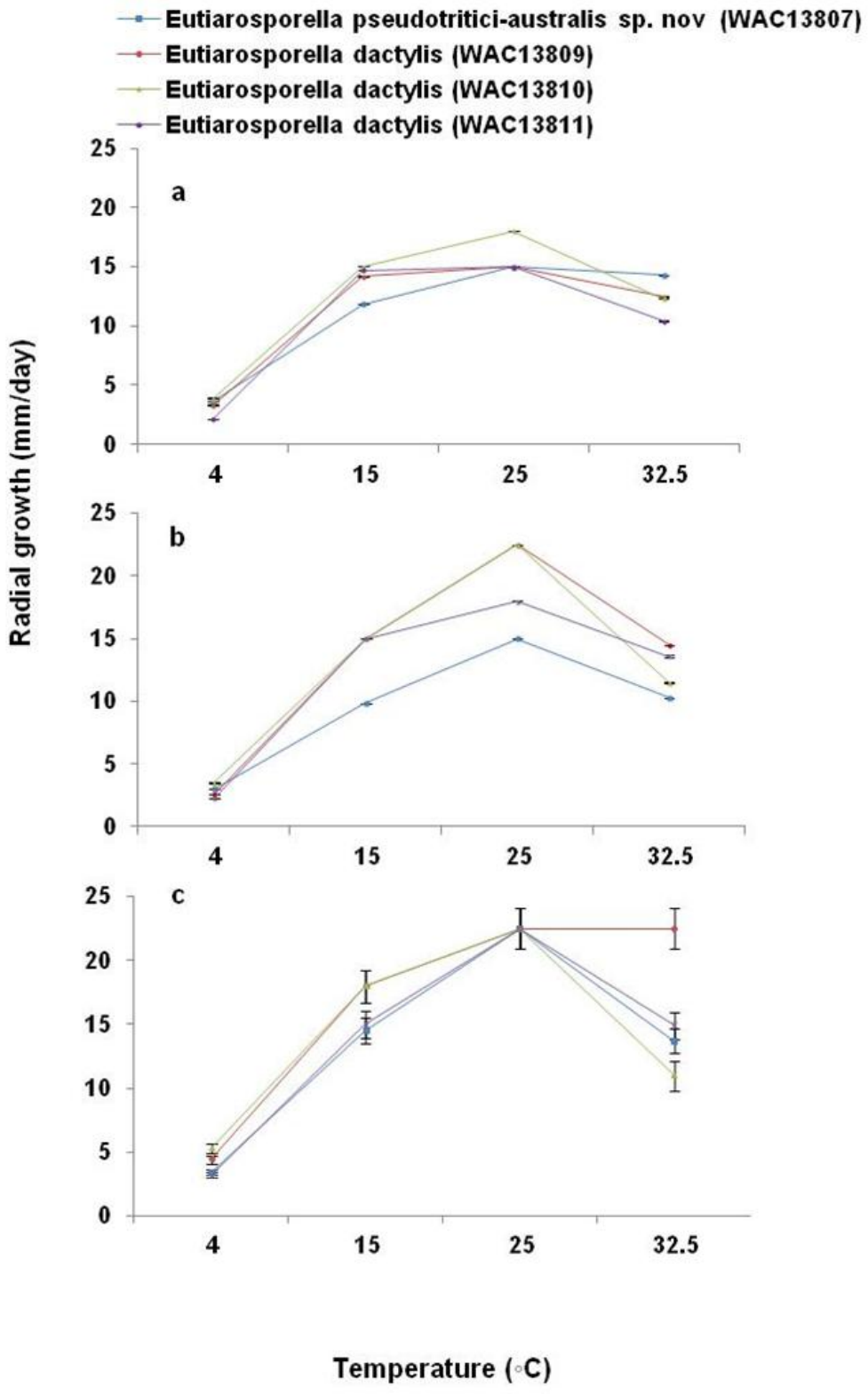


Figure 4.8 Mean radial growth rate (mm/d +/- SE) rates of one isolate (WAC13807) of *Eutiarosporella pseudotritici-australis* sp. nov. and three isolates (WAC13809, WAC13810, WAC13811) of *E. dactylis* on (a) wheat dextrose agar, (b) barley dextrose agar, and (c) on canola dextrose agar, at different temperatures. Bars indicate standard errors of the mean.

4.3.3 Phylogenetic Analysis

The ITS sequences for all isolates were aligned to members of Botryosphaeriaceae (Fig 4.9 and Appendix 8). According to this phylogeny, four isolates of *E. pseudotritici-australis* sp. nov. (WAC 13805, WAC 13806, WAC 13807 and WAC 13808) are resolved in the same clade as *Eutiarosporella tritici-australis* (Thynne *et al*. 2015), and three isolates (WAC 13809, WAC 13810 and WAC 13811) into the *Tiarosporella dactylis* clade (Thambugala *et al*. 2014).

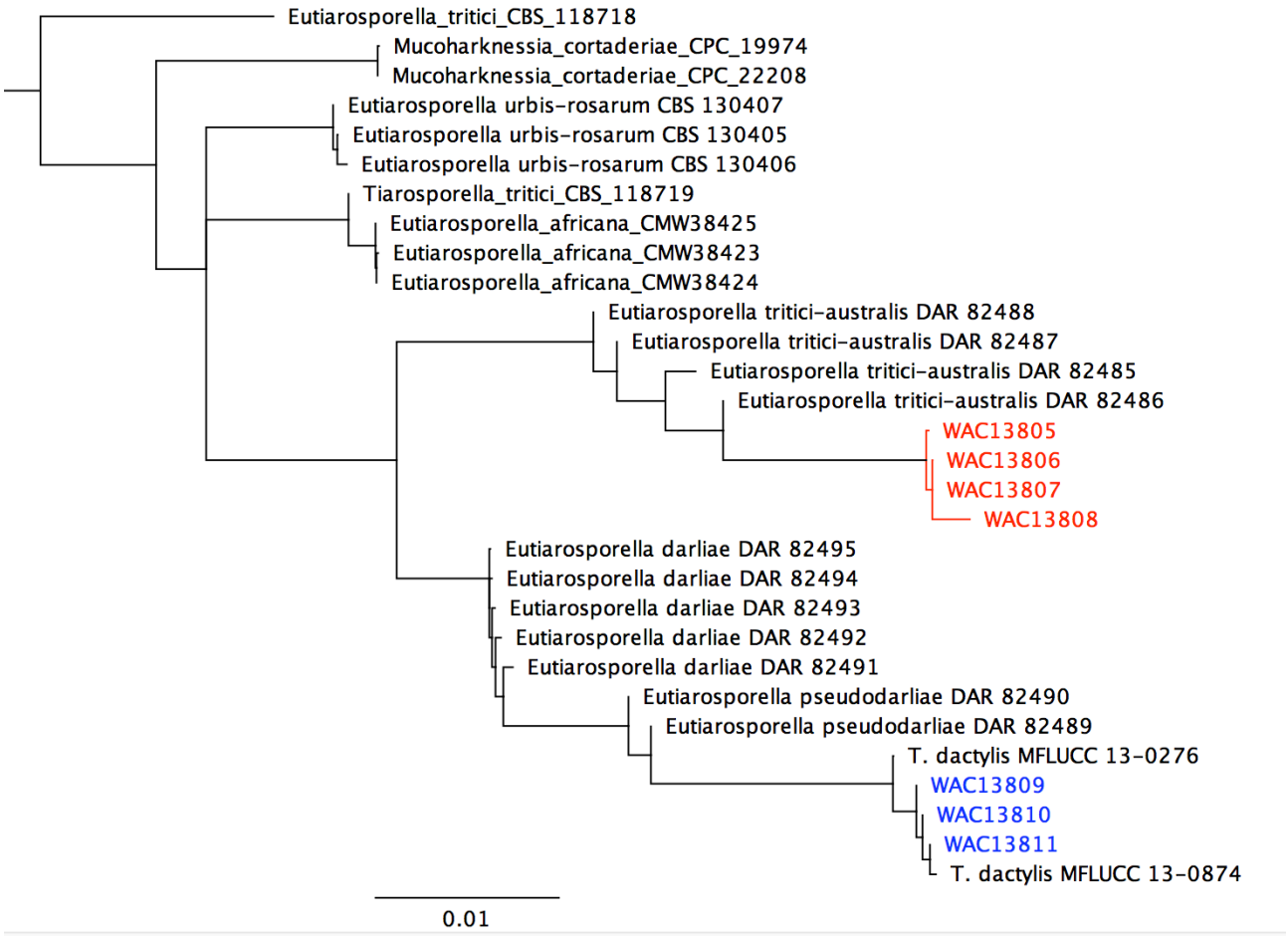


Figure 4.9 The relationship of the seven Western Australian *Eutiarosporella* isolates with other species of *Eutiarosporella* and *Tiarosporella*. Red colour indicates the four isolates that represent a putative new species (*Eutiarosporella pseudotritici-australis* sp. nov.). The blue indicates the three isolates that belong to *E. dactylis*.

4.4 *Eutiarosporella pseudotritici-australis* sp. nov. taxonomy

Taxonomy: *Eutiarosporella pseudotritici-australis* sp. nov. E. Barkat, G. Hardy & K. Bayliss, sp. nov. 2015.

Culture collection number: Holotype WAC13807; extype WAC13808.

Etymology**:** Name refers its closest known relative *Eutiarosporella tritici-australis* and with the country in which it was isolated.

Type species**:** *Eutiarosporella pseudotritici-australis* sp. nov. (E. Barkat, G. Hardy & K. Bayliss, sp. nov. wheat grain, 2012-2014).

Specimens examined: Wheat grains collected from Cooperative Bulk Handling (CBH) and from Western Australian farms in 2012- 2014.

Commentary**:** Based on phylogenetic analyses, isolates of *Eutiarosporella pseudotritici-australis* sp. nov. are most closely related to *Eutiarosporella tritici-australis* (Thynne *et al.* 2015), which was isolated from *Triticum aestivum* in Australia. It differs from *E. tritici-australis* in the morphology of conidiogenous cells and conidia which are all smaller than the conidiogenous cells and conidia of *Eutiarosporella tritici-australis*.

4.5 Discussion

The purpose of this study was to identify the species of *Eutiarosporella* associated with stored wheat. Four isolates belonged to a previously undescribed species of *Eutiarosporella* now named *Eutiarosporella pseudotritici-australis* sp. nov., whilst three isolates belonged to *E. dactylis.* Both *Eutiarosporella pseudotritici-australis* sp. nov. and *Eutiarosporella dactylis* are easily distinguished from morphologically similar *Eutiarosporella* and *Tiarosporella* species on the basis of sequence data, and by a range of morphological criteria.

The new species of *Eutiarosporella* was compared to published data for *Eutiarosporella pseudodarliae. E darliae, E. tritici-australis and E. paludosa* and was named *E. pseudotritici-australis* sp. nov. based on it being a sub-clade of *E. tritici-australis* described by Thynne *et al*. (2015). *E. tritici-australis* is a novel Australian wheat-infecting *Eutiarosporella* sp. (Thynne *et al*. 2015) that causes white grain disorder on wheat in the field. The second species identified, *Eutiarosporella dactylis,* was reported from the same host (wheat) as *Eutiarosporella pseudodarliae* that was also described by Thynne *et al*.

(2015), and falls in to a sub-clade of the latter species. Interestingly, although ITS analysis placed the isolates used in the current study with *E. dactylis* morphologically the condia differed considerably from the description of Thambugala *et al.* (2015). Briefly, in the current study the condia were rough walled and clavate and ovoid to fusoid in shape, whilst in Thambugala *et al.* (2015) they are described as smooth walled and ovoid, straight and oval in shape. Based on classical taxonomy, the isolates between the two studies would not be considered the same species. Therefore, future studies should examine more gene regions to determine whether they are the same species or not.

Species of *Tiarosporella* and *Eutiarosporella* have traditionally been associated with members of Poaceae (Sutton and Marasas 1976, Nag Raj 1993, Thambugala *et al.* 2014), although recent studies have also reported *T. urbis-rosarum* in healthy tissue of Acacia karroo (Fabaceae) (Jami *et al*. 2012; 2014) and *T. graminis* on hosts from the Zygophyllaceae and Asteraceae (Jami *et al*. 2012). *Tiarosporella paludosa* was reported from submerged wood from the Clohesy River in north Queensland in Australia (Hyde 1993). Three new species, *Eutiarosporella tritici-australis, Eutiarosporella darliae,* and *Eutiarosporella pseudodarliae* were only recently identified and characterised from wheat in Australia (Thynne *et al*. 2015). Sequences confirming the identity of *Tiarosporella* and *Eutiarosporella* spp. have been limited to isolates collected from South Africa (Jami *et al*. 2014) including the species *T. madreeya, T. tritici* (now *Eutiarosporella tritici*), and *T. graminis* (now *Marasasiomyces karoo*) (Crous *et al*. 2015), Thailand (Thambugala *et al*. 2014) and Australia (Thynne *et al*., 2015). In the present study, *Eutiarosporella pseudotritici-australis* sp. nov. and *E. dactylis* were both observed on stored wheat grain and confirmed by sequence analysis and this appears to be the first report of either species on stored grain.

Currently, very little is known about the biology, ecology or pathology of Eutiarosporella species that are found in Australia; however, they have not yet been shown to cause significant damage during postharvest storage. This study indicated that there was variation in growth rates between isolates on wheat dextrose agar, barley dextrose agar and canola dextrose agar. These growth rate variations could be due to different grains used as basal media (Adesemoye and Adedire 2005).

It is also not known how long they have been present, or if they are new introductions. If they are recent introductions, then they could well become postharvest pathogens in time if conditions are suitable. One feature that sets *Eutiarosporella* apart from other endophytes is the potential of some species to become aggressive pathogens when plants become stressed (Sakalidis *et al*. 2011), consequently they are potentially both pre and postharvest pathogens on wheat. Thynne *et al*. (2015**)** recently reported that three species of *Eutiarosporella* (*Eutiarosporella tritici-australis*, *Eutiarosporella darliae* and *Eutiarosporella pseudodarliae*) are associated with white grain disorder on wheat. These symptoms were also recorded in the late of 1990s in Queensland (Kopinski and Blaney 2010) and in Victoria (VIC) and South Australia (SA) in 2010 **(**Thynne *et al*. 2015**).** Consequently, future work should concentrate on *E. dactylis* and *E. pseudotritici-australis* sp. nov. as potential pre- and postharvest pathogens of wheat in Australia and elsewhere. It would also be beneficial to look at a larger number of isolates to determine if there are differences between isolates with regards to their biology, ecology and pathology in stored grain and in wheat crops.

The present study indicated that there was variation between isolates in growth rates on PDA, MEA and CMA at different temperatures. Isolates that had faster growth rates might cause spoilage in postharvest grain earlier than slower isolates due to physiological differences influencing rates of sporulation or growth of mycelia. Thynne *et al*. (2015) reported that differences in growth on media could have application as a tool for discriminating between isolates, as an additional measurement to morphological and molecular approaches. Further work is required to understand the potential difference in pathogenicity of the isolates. It would be useful to apply further molecular analyses to both *Eutiarosporella* species to determine if there is any genetic variation between the isolates.

Chapter 5: Analysis of volatile organic compounds from stored wheat grain and postharvest fungi using solid phase microextraction and gas chromatography mass spectrometry

5.1 Introduction

Fungal growth in food and animal feed can lead to considerable spoilage, which can be manifested through nutritional losses, development of mycotoxins and potentially allergenic spores, and unpleasant odours (Filtenborg *et al*. 1996). Volatile organic compounds (VOCs) can be released after harvest and during storage, either directly from the commodity, or as a result of insect and microbial infestation. The contents of volatiles are related to the history of the commodity (pre-harvest conditions and treatments during storage) (Girotti *et al*. 2012) and the volatile compounds produced by grain and fungi can be used to distinguish fungal contamination in stored grain (Nieminen *et al*. 2008).

An extensive range of volatiles are produced by cereal grains including alcohols, esters, aldehydes, ketones, alkanes, alkenes, furans, lactams, phenols, pyrazines, and pyrroles (Kaminski and Wasowicz 1991). To date, the main volatiles recorded in grain include 3-methyl-1-butanol, 1-octen-3-ol, 2-methylfuran and 3-octanone, as determined by gas chromatography-mass spectrometry (GC-MS) and sensory analysis (Börjesson *et al*. 1994; Schnürer *et al*. 1999). Volatile profiles also vary with microorganisms. For example, using a mass spectrometry-based e-nose system, the volatile profile of mould contamination of bakery products has been successfully used in the discrimination of moulds such as *Eurotium*, *Penicillium* and *Aspergillus* species (Vinaixa *et al*. 2004).

Previous studies have demonstrated that it is possible to use headspace analyses combined with a gas chromatography flame ionization detector and gas chromatography-mass spectrometry to accurately determine and quantify the volatiles on wheat produced by different mycotoxigenic spoilage fungi such as *Penicillum verrucosum*, *Aspergillus ochraceus* and *A. carbonarius* (e.g. Sahgal *et al*. 2007; Börjesson *et al*. 1994 and Jelen *et al*. 2003). Consequently, this methodology seems to be very promising for screening for the presence of fungal species in stored grain. The aim of this study was to determine if specific VOCs produced by different fungal species could be used to determine the presence of fungi in wheat grain during storage. The hypotheses were (a) that VOCs produced by storage fungi can be used for early detection of fungal contamination in stored grain, prior to the development of visible signs and symptoms, and (b) VOCs can be released from commodities after harvest and during storage, directly from grain itself or as a result of microbial infestations.

5.2. Materials and Methods

5.2.1 Preparation of wheat samples from farms to measure volatiles

Twenty-three samples of wheat grain were collected from selected farms from across Australia as described in Chapter 3 and each was divided into two sub-samples for VOC analysis – comprising the original grain which had its moisture content determined on arrival, and grain that was adjusted to 15% moisture, the moisture content was adjusted by placing the wheat in a sealed flask (3L) which was sealed and allowed to eqilabrate to 25°C for three days before the addition of distilled water, and confirmed with an electronic moisture meter (Graintec HE 50 electronic moisture meter, Graintec Pty Ltd, Toowoomba, Australia). The samples were directly placed in water bath to extract the volatiles. Three different treatments were used to determine the presence of VOCs in grain samples. Treatment 1 was 'original' grain from the farms (see Chapter 3). Treatment 2 was the 'original' grain adjusted to 15% moisture content by adding a calculated volume of distilled water into a sealed flask (3L) at 25C for 1 week. Treatment 3 was wheat grain obtained from Cooperative Bulk Handling, Western Australia, and adjusted to either 11% and 15% moisture content, before being gamma-irradiated for one week at 30, 000 GY and then a small sample placed on PDA for 5-7 days to confirm sterility. There were two replicate flasks for each treatment to give a total of 96 flasks (46 flasks for each of Treatment 1 and 2 and four flasks for Treatment 3). Gamma-irradiated controls were included to distinguish any difference between the farm samples and known sterile grain.

5.2.2 Preparation of wheat samples spiked with known fungi to measure volatiles

Ten fungal species isolated from stored grain samples (Chapter 3) and one isolate of *Fusarium graminearum* (WAC 11387) obtained from the Department of Agriculture and Food Western Australia culture collection were used in this Experiment (Table 5.1). The ten fungal isolates had been sequenced using ITS to confirm identity (Chapter 3). The *Fusarium graminearum* isolate is known to produce mycotoxins (Tan *et al*. 2011). These 11 isolates were grown on PDA at 25°C for 7 days in the dark before 5 plugs of $5mm^2$ in size were taken from the edge of the colonies and used to inoculate gamma-irradiated grain (adjusted to 15% moisture content) and the flasks were incubated at 25°C in the dark for 1, 2, 3, 7 and 10 days prior to the VOCs being analysed. The control was gamma-irradiated

grain at 15% moisture content inoculated with plugs of sterile PDA. There were two replicates for each isolate to give a total of 22 flasks, with 10 flasks as controls.

Table 5.1 Isolates used to spike gamma-irradiated grain for VOCs production.

Species	WAC Code	Region
Alternaria alternata	13842	NSW
Alternaria infectoria	13850	WA
Alternaria infectoria	13852	NSW
Alternaria infectoria	13851	WA
Auerobasidium sp.	13824	WA
Cladosporium herbarum	13833	WA
Cladosporium cladosporioides	13838	WA
Cladosporium cladosporioides	13841	NSW
Fusarium graminearum	11387	WA
Penicillium cordubense	13831	SA
Penicillium dipodomyicola	13832	NSW

5.2.3. Extraction of volatiles

Each of the grain samples (50 g) was placed in a 100 mL Erlenmeyer flask (Fisher Scientific, Quickfit, UK; Cat. NoFE 100/3) equipped with a cone/screw-thread adapter (Crown Scientific, Code ST 5313) with a 7/16" blue septum (Grace Davison Discovery Sciences, Cat. No. 6518). The measured volume of each Erlenmeyer flask and inlet system was calculated from the weight of water required to fill the flask. The flasks were placed for 3 hours in a 45°C water bath prior to extracting of the volatiles. Two fibres (SPME fibre 50/30 μm polydimethylsiloxane (PDMS; Cat. No. 57348-U from Analytical Sigma-Aldrich), selected according to Qiu *et al*. (2014a) and conditioned prior to use in accordance with the manufacturer's recommendations were inserted into the headspace of the flask containing the samples at the end of the defined extraction time for three hours. The fibre holder was then removed from the extraction flask and inserted into the injection port of the GC-FID. The fibre was extended into a GC-FID inlet where sample components were desorbed.

5.2.4. Optimisation of measurement conditions

Optimal headspace solid-phase microextraction gas chromatography conditions following SPME parameters were used according to Qiu *et al*. (2014a). Briefly, for the optimisation of the standards, the appropriate volumes of each standard were added into a sealed 250 mL bottle. After 5 min extraction with the fibre 50/30µm DVB/CAR/PDMS at room temperature (24±1ºC), the fibre was injected into the gas chromatograph at the injector temperature of 250ºC for desorption of >3 min.

5.2.5. Gas chromatography-flame ionization detector (GC-FID) analysis

The VOC analysis from all samples was performed on an Agilent 6890 Gas Chromatograph manufactured by Agilent Technology (Palo Alto, CA, USA) and a Flame Ionization Detector (FID; Hewlett Packard 6890 series) was used to analyse the volatile profiles extracted by HS-SPME. The columns used in this experiment were a Stabilwax® polar column (Dimensions: 30 m × 0.25 mm × 0.25 µm film thickness, ZB-WAX, Cat. No. #10623) and an Rxi®-5ms non-polar column (Dimensions: 30 m × 0.25 mm × 0.25 µm film thickness, RESTEK, Cat. No. #13423). Helium was used as the carrier gas at a constant speed of 40 mL min-1 in the split-less mode. The column temperature program was set at 45ºC for 5 min, then increased by 5ºC min-1 to 250ºC and held for 5 min and the GC-FID instrument was operated under the split-less mode. The helium inlet pressure was controlled at a constant flow of 1.0 ml min-1. The ionization potential was set at 70 eV, and scanning was performed from 35 to 500 atomic mass units at a rate of 3.15 scans sec-1. The retention Kovates-index (Kovats, 1958) was calculated by using Pherobase (i.e., the relative retention values based on a scale defined by the elution of a series of n-alkanes) obtained freely online from the website www.pherobase.com/database/kovats/kovats-index.

5.2.6. Gas chromatography-mass spectrometry (GC-MS) analysis

In addition to the VOC analysis using GC-FID, GC-MS was used to identify specific compounds produced by two species of *Alternaria* (*A. infectoria* WAC13850 and *A. alternata* WAC13842) and *Cladosporium herbarum* (WAC13833). These three isolates were selected because they were recovered frequently from grain samples (Table 3.3, Chapter 3). An Agilent 6890 gas chromatograph equipped with the 30 m × 0.25 mm × 0.25 µm Stabilwax® polar column that was coupled to an Agilent 5973 Network mass selective detector (MSD) with an Agilent ChemStation was used to identify the separated VOCs. The

column temperature program was set the same as GC-FID. The injection port (splitless mode), interface and MS source temperature were 250ºC and 230ºC, respectively. The volatiles were identified by comparison of the mass spectrum with the NIST08 mass spectra library together with retention indices (NIST08 mass spectral search program for the NIST/EPA/NIH mass spectral library version 2.0F), built in 2008).

5.2.7 Statistical analysis

Unique VOCs were identified using GCALIGNER 1.0 and GCKOVATS according to Dellicour and Lecocq (2013). The difference between the original and moistened farm samples and the spiked grain were analyzed for preliminary data using a comparison matrix of chemical data obtained by GC/FID. The alignment algorithm was based on the comparison between the retention times of each detected compound in a sample.

5.3 Results

5.3.1 Analysis and identification of volatile organic compounds in farm samples

From the 23 original farm samples tested there was variation in the production of volatiles among samples from the same state based on retention time (Table 5.2). Individual samples from WA, QLD and SA produced peaks at 13.24 min, one sample of grain from SA produced a specific peak at 15.84 min and one WA sample was found to produce a peak at 29.85 min whereas grain from VIC and NSW did not produce specific VOCs at any of these retention times (Table 5.2). One VOC at 40.89 min was identified in one sample of grain from WA and five samples from SA, and at 40.96 min there was a peak found in two samples of WA grain, two from NSW and one from Victoria. There were no volatiles found on gamma-irradiated grain.

When the farm grain samples were adjusted to 15% moisture content and re-measured, unique peaks specific to individual samples were found from grain from QLD (at 10.05 min) and WA 9 at 13.24 and 15.84 min) (Table 5.3). At 26.4 min two samples from WA and one sample from NSW shared a VOC. Another shared VOC was produced in the headspace of individual SA and QLD farm samples at 40.89 min (Table 5.3). Three sampes of grain from SA and one from Victoria produced a specific peak at 40.96 min. Gamma-irradiated wheat did not produce any unique peaks.

A total of 57 possible chemicals were detected in original grain (Treatment 1) and grain at 15% moisture content (Treatment 2) based on retention time of the peaks (Table 5.4).

Table 5.2. Volatile organic compounds (VOCs) detected by GC-FID in the headspace of samples from farm grain at original moisture content collected from five states across Australia included Western Australia (WA), South Australia (SA), New South Wales (NSW), Victoria (VIC), and Queensland (QLD).

RT	WA1 10%	WA2 11%	WA3 11%	WA4 9%	WA5 10%	WA6 10%	WA7 10%	WA8 9%	SA1 11%	SA2 10%	SA3 9%	SA4 13%	SA5 9%	SA6 9%	NSW1 11%	NSW2 11%	NSW3 12%	NSW4 14%	NSW5 11%	VIC2 12%	VIC2 12%	VIC3 12%	QLD 10%	Control
10.05	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13.24	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-
15.84	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
26.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29.85	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
40.89	-	-	-	-	-	-	-	+	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
40.96	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-

RT, retention time; VOCs produced by original grain (+) and (-) not detected, % moisture content of the grain.

Table 5.3. Volatile organic compounds (VOCs) detected by GC-FID in the headspace of samples from original farm grain adjusted to 15% moisture content from five states across Australia and included Western Australia (WA), South Australia (SA), New South Wales (NSW), Victoria (VIC) and Queensland (QLD).

RT	WA1 10%	WA2 11%	WA3 11%	WA4 9%	WA5 10%	WA6 10%	WA7 10%	WA8 9%	SA1 11%	SA2 10%	SA3 9%	SA4 13%	SA5 9%	SA6 9%	NSW1 11%	NSW2 11%	NSW3 12%	NSW4 14%	NSW5 11%	VIC2 12%	VIC2 12%	VIC3 12%	QLD 10%	Control
10.05	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
13.24	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15.84	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
26.4	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
29.85	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
40.89	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-
40.96	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	+	-	-	-

RT, retention time; VOCs produced by grain at 15% (+) and (-) not detected.

Table 5.4. Possible chemicals for each of the seven retention times detected by GC-FID in the headspace of samples from original farm grain and farm grain adjusted to 15% moisture content.

RT (min)	Possible chemicals	CAS No	Formula
10.05			
	(E,E)-2,4- Hexadienal	142-83-6	C_6H_8
	2,4-Dimethyloctane	4032-94-4	$C_{10}H_{22}$
	2-Ethylpyridine	100-71-0	C_7H_9N
	4,5-Dimethylthiazole	3581-91-7	C_5H_7NS
	Heptanal	111-71-7	$C_7H_{14}O$
13.24			
	(4-Fluorophenyl)-methanamine	659-41-6	$C_7 H_8 F N . Cl H$
	1,3,5-trimethylbenzene	108-67-8	$C_6H_3(CH_3)3$
	(E,Z)-2,4-Heptadienal	4/02/4313	$C_7H_{10}O$
	1-Isopropenyl-4-methylenecyclohexane	499-97-8	$C_{10}H_{18}$
	3-Methylbutyl 2-methylpropionate	3/01/2050	$C_9H_{18}O_2$
	Benzaldehyde	100-52-7	C_7H_6O
	Butyric acid	107-92-6	$C_4H_8O_2$
	Ethyl 2-(methylthio)-acetate	4455-13-4	$C_5H_{10}O_2S$
	Ethyl 2-bromobutyrate	66025-42-1	$C_6H_{11}BrO_2$
	Ethyl hexanoate	123-66-0	$C_8H_{16}O_2$
	Ethyl propyl disulfide	30453-31-7	$C_5H_{12}S_2$
	Isopropyl trichloroacetate	3974-99-0	$C_5H_7C_{l3}O_2$
	Methyl 4-methylhexanoate	2177-82-4	$C_8H_{16}O_2$
	tert-Butyl 3-chloropropanoate	55710-80-0	$C_7H_{13}ClO_2$
15.84			
	1,2,4-Trithiapentane	-	$C_2H_6S_3$
	2,3,4-Trimethylfuran	10599-57-2	$C_7H_{10}O$
	2-Ethyl-3-methylpyrazine	15707-23-0	$C_7H_{10}N_2$
	4-Methylphenol	106-44-5	C_7H_8O
	4-Penten-2-yl bromoacetate	-	$C_7H_{11}BrO_2$
	4-tert-Butyltoluene	98-51-1	$C_{11}H_{16}$
	6-Ethyl-2-vinylpyrazine	32736-90-6	$C_8H_{10}N_2$
	6-Ethyl-3-hydroxy-2-methyl-4H-pyran-4-one	8/11/4940	$C_8H_{10}O_3$
	6-Methyl-3,5-heptadien-2-one	1604-28-0	$C_8H_{12}O$
	Benzyl formate	104-57-4	$C_8H_8O_2$
	tert-Pentylbenzene	2049-95-8	$C_{11}H_{16}$
26.4			
	(Z)-3-Dodecen-1-ol	32451-95-9	$C_{12}H_{24}O$
	2,3,6,7,8,8alpha-Hexahydro-1,4,9,9-tetraethyl-1H-3alpha,7-methanoazulene	560-32-7	$C_{15}H_{24}$
	2beta-Hydro-6alpha-methoxy-trans-decalin	-	$C_{11}H_{20}O_2$

Table 5.4 (continued). Possible chemicals for each of the seven retention times detected by GC-FID in the headspace of samples from original farm grain and farm grain adjusted to 15% moisture content.

RT (min)	Possible chemicals	CAS No	Formula
26.4	3,4,5-Trichloroanisole	54135-82-9	$C_7H_5Cl_3O$
	6-Methyl-1,2,3,4,5,6-hexahydro-(7H)- cyclopentapyridine-7-one	-	$C_9H_{15}NO$
	Acora-4,10-diene	-	$C_{15}H_{24}$
	Cadina-3,5-diene	-	$C_{15}H_{24}$
	Dimethyl 2,5-dichloro-4-bromo-3-oxopentanoate	-	C7H8BrCl2 O_3-
	Furan-2-carbaldehyde	98-01-1	$C_5H_4O_2$
	Muurola-4,11-diene	-	$C_{15}H_{24}$
	p-Chlorobenzylidene-propyl-amine	-	$C_{10}H_{12}$ CIN
	p-Ethyl-dimethyl hydrocinnamic aldehyde	-	$C_{13}H_{18}O$
29.85			
	(Z)-2,6-Dimethoxy-4-(prop-1-enyl)-phenol	-	$C_{11}H_{14}O_3$
	(Z)-5-Tridecen-2-yl acetate	-	$C_{15}H_{28}O_2$
	11-Dodecenyl acetate	35153-10-7	$C_{14}H_{26}O_2$
	2beta-Hydroxy-6alpha-cyanide-trans-decalin	-	$C_{11}H_{17}NO$
	Benzophenone	9133-55-5	$C_{13}H_{10}O$
	Cedrenol	28231-03-0	$C_{15}H_{24}O$
	Globulol	51371-47-2	$C_{15}H_{26}O$
40.89			
	(E)-14-Hexadecen-1-ol	-	$C_{16}H_{32}O$
	(E)-2,5-Dimethyl-3-styrylpyrazine	-	$C_{14}H_{14}N_2$
	1-Methyl-4-(1-hydroxy-1-methylethyl)-benzene	1197-01-9	$C_{10}H_{14}O$
	7,12-Dimethylhexadecane	-	$C_{18}H_{38}$
	7,12-Dimethyloctadecane	-	$C_{20}H_{42}$
	N,N-Dimethyl-N-octyl-benzamidine	-	$C_{17}H_{28}N_2$
40.96			
	(Z)-9-Methylhexadecenoate	-	$C_{17}H_{32}O_2$
	9-Tigloylretronecine	-	$C_{13}H_{19}NO_3$

5.3.2 Volatile organic compounds produced on grain spiked with known fungi

For the *Alternaria* isolates, three specific volatile peaks were produced which varied in the time of detection (Table 5.5). *A. alternata* (WAC13842) produced a peak at 13.24 min at 1, 2 and 7 dpi and *A. infectoria* (WAC11850) produced the same peak after 7 dpi. A second specific peak was produced at 15.84 min after 7 dpi by *A. alternata* WAC13842 and 10 days and also this peak was identified in two of the *A. infectoria* isolates (WAC11850 and WAC11852) from 1 and 2 dpi (Table 5.5). A third peak at 26.4 min was detected in the headspace of *A. alternata* WAC 13842 after only 1 dpi and also in *A. infectoria* WAC 11852 at 7dpi. *A. infectoria* isolate WAC13851 did not produce any unique peaks and neither did the controls.

Five specific VOCs were detected across the three isolates of *Cladosporium*, two isolates of *Penicillium* and one isolate each of *Aureobasidium* and *Fusarium* (Table 5.6). At 10.05 minutes, a peak unique to *Cladosporium cladosporioides* was detected in both isolates, but nine days apart. The two *Penicillium* spp. both had a peak at 20.1 min that was present from 1 dpi until at least 7 dpi, and both also had a second peak at 40.89 min at 1 dpi. Similarly two of the *Cladosporium* isolates produced a specific peak at 29.85 and also 40.96 at 2 dpi. The peak at 40.96 min was also found to be common to *Penicillium dipodomyicola, Aureobasidium* sp. and *Fusarium graminearum* but varied in the time of detection from 1 dpi to 10 dpi. (Table 5.6).

There was a reasonable correlation between the peaks observed in the spiked samples and those in the farm samples. For example, samples spiked with *Cladosporium cladosporioides* (WAC13838 and WAC13841) produced a specific peak at 10.05 min and this peak was also observed in the original grain sample from QLD (Table 5.3). A specific peak in grain spiked with *A. alternata* (WAC13842) and *A. infectoria* (WAC11850) at 13.24 min, was observed in the original grain from WA and the grain at 15% from WA, SA and QLD (Tables 5.2 and 5.3) and peaks commonly produced by all *Alternaria* species (Table 5.5) at 15.84 min and 26.4 respectively were observed in original grain from WA (Table 5.2) and grain from SA at 15% (Table 5.3). *Penicillum* species (WAC 13831 and WAC 13832) produced a specific peak at 40.89 min, and this was observed in grain from WA and SA on arrival and grain at 15% from SA and QLD. A common peak at 40.96 min

found in the headspace of grain spiked with *Cladosporium* spp., *Aureobasidium* sp., *F. graminearum* and *P. dipodomyicola* was observed in original grain from SA, QLD and VIC, and grain at 15% from WA, NSW and VIC. (Tables 5.2 and 5.3).

Table 5.5 Volatile organic compounds detected by GC-FID from 15% moisture content grain spiked with *Alternaria alternata* WAC13842 or one of three isolates of *A. infectoria* (WAC11850, 113851 or 113852) from 1 to 7 days post-inoculation. There were only three retention times detected in the spiked grain samples, and their time of detection (post-inoculation) varied among isolates.

RT	Kovats index	*A. alternata*		*A. infectoria*		Control
		WAC 13842	WAC 13850	WAC 13851	WAC 11852	
13.24	996	1,2,7	7	-	-	-
15.84	1076	7	1,2,3,7,10	-	1,2	-
26.4	1076	1,2,3	-	-	7,10	-

RT, retention time; VOCs produced by *Alternaria alternata*, and three isolates of *A. infectoria* (+) and (-) not detected; Numbers indicate days on which detected.

Table 5.6 GC-FID volatile organic compounds detected by GC-FID from 15% moisture content grain spiked with *Aureobasidium* sp., *C. herbarum*, *C. cladosporioides*, *F. graminearum*, *P. cordubense* and *P. dipodomyicola* colonized grain at 15% moisture content. There were only five retention times detected in the spiked grain samples, and their time of detection (post-inoculation) varied among isolates.

RT (min)	Kovats index	*C. herbarum*	*C. cladosporioides*		*P. cordubense*	*P. dipodomyicola*	*Aureobasidium* sp.	*F. graminearum*
		WAC 13833	WAC 13838	WAC 13841	WAC 13831	WAC 13832	WAC13824	WAC11387
10.05	906	-	1	10	-	-	-	-
20.1	1217	-	-	-	1,2,3,7,10	1,2,3,7	-	-
29.85	1604	2,3,7	-	2	-	-	-	-
40.89	1887	-	-	-	1,3,7,10	1,2	-	-
40.96	1888	2	-	2	-	10	1,2,3,7	1,2,3,7,10

RT, retention time; Grain colonized by fungi represented VOCs from different incubation time (+) and (-) not detected; Numbers indicate days on which detected.

5.4.3 GC-MS identification of compounds from *Alternaria alternata, A. infectoria* and *Cladosporium herbarum*

Based on GC/FID results, the volatiles produced by three fungal species (*A. alternata* WAC13842, *A. infectoria* WAC13850 and *C. herbarum* WAC13833) were further characterized by comparing mass spectra with library spectra and determining chromatographic retention indices gas chromatography-mass spectrometry (Table 5.7). The largest peak found in the headspace of *A. alternata* was idenitifed as Cyclooctasiloxane, hexadecamethyl- (Table 5.7; Figure 5.1.a), and it was not produced in the presence of the other two fungi. Pentadecane was produced by *A. infectoria* (Table 5.7; Figure 5.2.b) and was unique to this fungus. Naphthalene was only identified in the headspace of *C. herbarum* (Table 5.7; Figure 5.3.c).

Table 5.7. Volatile organic compounds collected from irradiated grain inoculated with *A. alternata, A. infectoria* or *C. herbarum* and identified using gas chromatography-mass spectrometry (GC-MS) analysis.

Peak	Peak area %	*Alternaria alternata* WAC13842	*Alternaria infectoria* WAC13850	*Cladosporium herbarum* WAC13833	Likely Compound	Match quality %
		+	-	-	Cyclooctasiloxane, hexadecamethyl-	
1	1.16					93.3
2	2.03	-	+	-	Pentadecane	48.1
3	0.10	-	-	+	Naphthalene	45.5

Volatiles produced by *A. alternata*, *A. infectoria* or *C. herbarum* (+) or not detected (-).

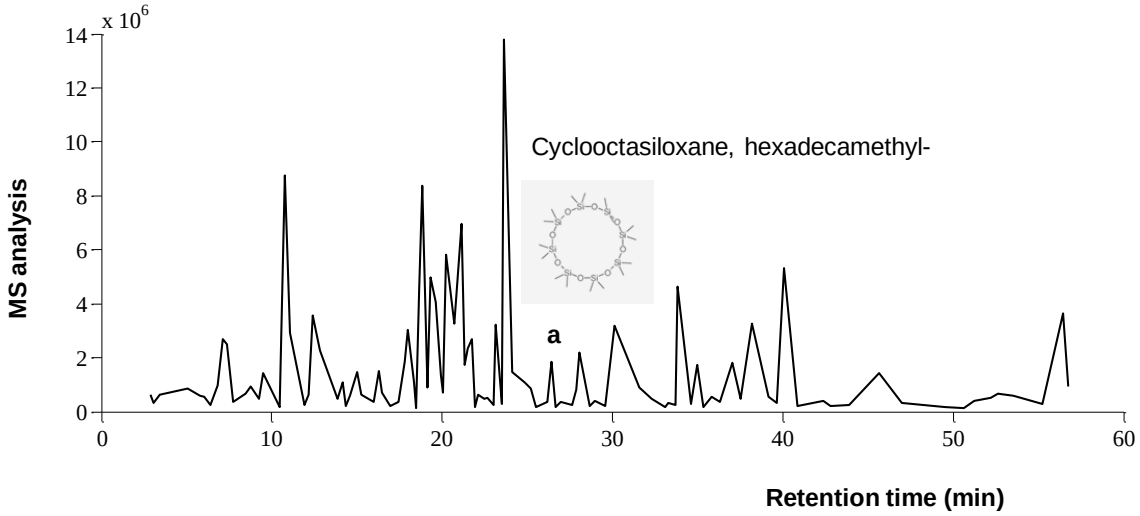


Figure 5.1 Chromatogram of GC-MS of the specific VOC Cyclooctasiloxane, hexadecamethyl- from gamma-irradicated grain samples colonised by *A. alternata* (WAC13842).

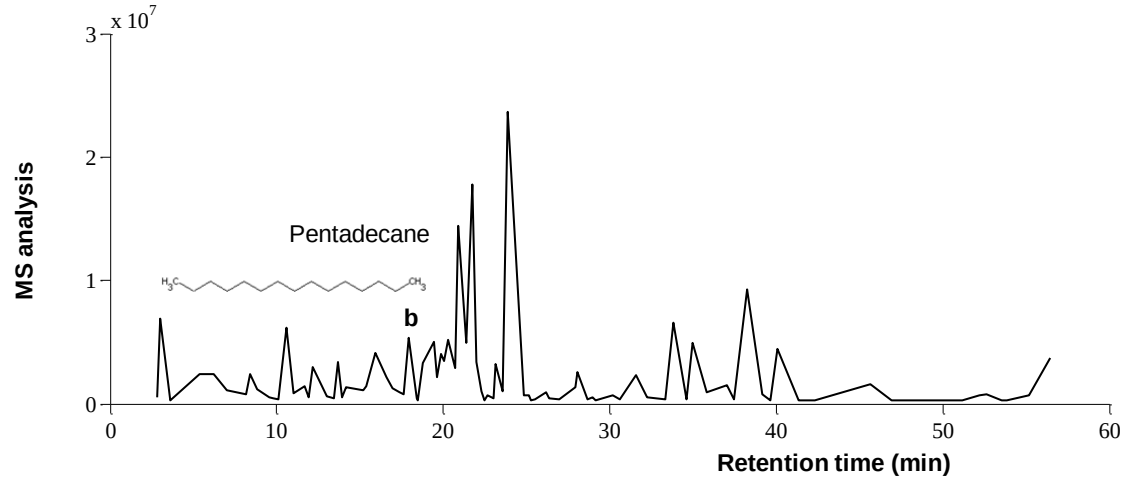


Figure 5.2 Chromatogram of GC-MS of the specific VOC Pentadecane from gamma-irradicated grain samples colonised by *A. infectoria* (WAC113850).

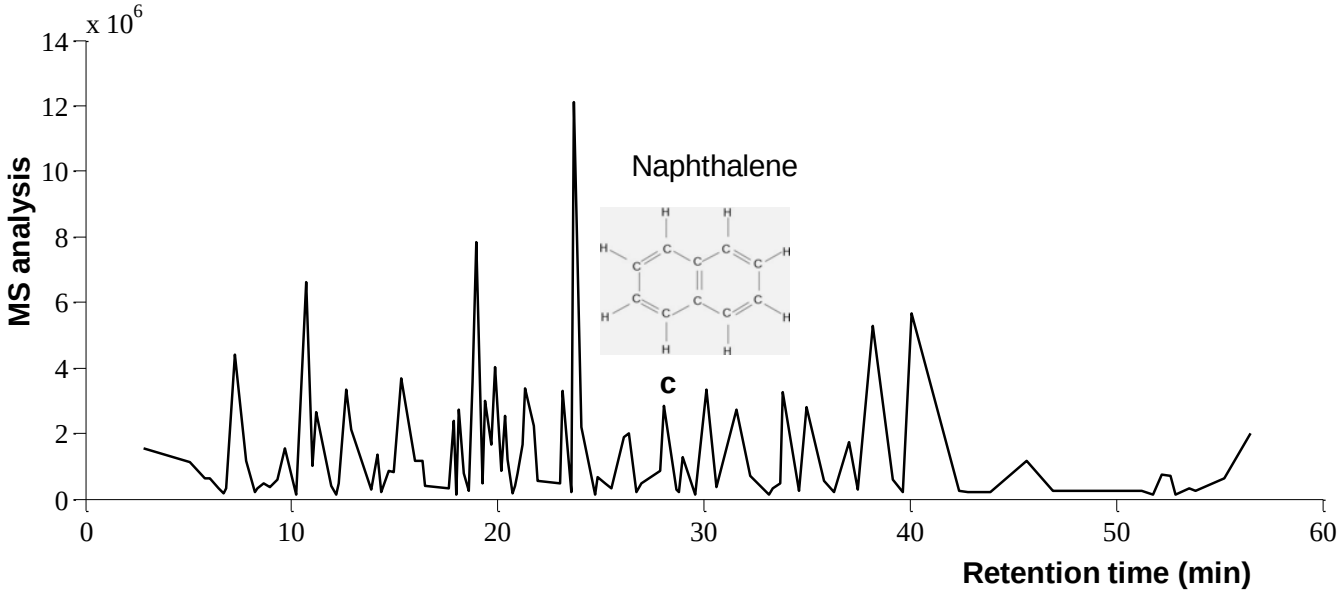


Figure 5. 3 Chromatogram of GC-MS of the specific VOC Naphthalene(c) from gamma-irradicated grain samples colonised by *C. herbarum* (WAC 13833).

5.4 Discussion

The results of this study confirm the hypothesis that VOCs can be used to determine the presence of fungi in storage facilities at an early stage of development. This study found that different volatiles are produced in grain samples from farms across Australia, however grains from QLD, VIC and NSW produced smaller amounts of VOCs compared to grain obtained from SA and WA. This result is contrary to expectations, given the diversity of fungi detected by diversity profiling, but does appear to correlate well with the traditional isolations. In general, it would be expected that the increase in temperatures (+0.91 °C above average) and decline in the rainfall pattern (10%) in NSW and Victoria (Bureau of Meteorology 2014) could affect the production of VOCs by storage fungi.

Another possible reason for this variation in VOC production between the states, could be due to the difference in the numbers of farms sampled from each state. The results suggest that the small grain samples might influence apparent differences in the configuration of volatile profiles between states. It could also be expected that different treatments used on farm storage facilities across states, might contribute to the differences in the volatiles produced in the different grain samples between states. Darby and Caddick (2007) reported

that different control methods (e.g. cleaning seeds, drying, aeration and chemical controls of pests) used on farms could influence the growth of fungi and in turn the production of VOCs. In the present study, fewer volatiles were produced on grain adjusted to 15% moisture content compared to those produced from the original farm samples. For example, moistened grain from NSW, WA and SA produced smaller amounts of VOCs compared to the original grain from the same regions. The variation in number of volatiles produced is probably due to the different moisture contents of the farm samples. Paolesse *et al*. (2006) indicated that water activity is a critical factor in the pattern of a chemical profile and Magan and Evans (2000) suggested that this could have a considerable impact on, and modify the significance of, individual volatile compounds produced by a specific species on temperate or tropical cereal grains. Another explanation of these findings could be due to different chemicals being produced in the headspaces, most likely due to the different fungal species present on the grain (De Lucca *et al*. 2012).

There were some correlations between volatile patterns and fungal growth across states (as indicated in Chapter 3). For example, specific peaks produced in spiked grain were also observed in the headspace of farm samples. This finding suggests that there is relationship between the VOCs produced and the fungi colonizing the grain, which might be useful to determine the presence of storage fungi in farm samples. Magan and Evans (2000) demonstrated that there are a range of VOCs produced by fungi when colonising grain, including 3-methyl-1-butanol, 1-octen-3-ol and other 8-carbon ketones and alcohols, however, none of these volatiles were found on the grain in this study. Likewise, Börjesson *et al*. (1989) identified a number of volatiles produced in the headspace of wheat spiked with *Aspergillus amstelodami*, *Aspergillus flavus*, *Penicillium cyclopium*, *and Fusarium culmorum* including 2-methylfuran, 2-methyl-I-propanol, and 3-methyl-I-butanol, which were not produced in the current study. The reason for the difference in the metabolites between this study and others could be due to the different methods used for collection of samples, extraction and analysis (Girotti *et al*. 2012). Although, not included in the present study, in future studies it will be useful to determine if there is a correlation between samples with VOCs and the climate where the sample was collected. However, to do this meaningfully will require many more samples from each region, and from different times of the year, and from different storage facilities.

Three fungal species were able to produce specific VOCs on sterile grain and they were Cyclooctasiloxane, hexadecamethyl- produced by *Alternaria alternata*, Pentadecane produced by *A. infectora* and Naphthalene produced by *Cladosporium herbarum*. Pentadecane has also been reported in cultures of *Aspergillus flavus* on cracked corn (De Lucca *et al*. 2012) whilst Naphthalene was also shown to be produced by *Aspergillus niger* on wheat (Harris *et al*. (1986). Cyclooctasiloxane, hexadecamethyl- was also produced by *Aspergillus terreus* isolated from soil (Rajalakshmi and Mahesh 2014). Further studies are required to develop knowledge of the types and amounts of VOCs present and their significance during postharvest storage in Australia. Whilst more isolates of each species are required to confirm whether the VOCs are unique or not to a species, it can be concluded that volatile fungal metabolites can be used to detect and quantify fungal growth in grain.

There is significant interest in being able to detect fungal spoilage specific volatiles at an early stage in stored grain, as this will assist in the timely management of stored grain that could be subject to spoilage and in turn substantial economic losses to the growers. The hypotheses of this study were confirmed, namely (a) that VOCs produced by storage fungi can be used for early detection of fungal contamination in stored grain, prior to the development of visible signs and symptoms, and (b) VOCs can be released from commodities after harvest and during storage, directly from grain itself or as a result of microbial infestations. Therefore, this study has confirmed that there is a potential to use VOCs for the early detection of spoilage fungi in grain storage facilities. In this study some VOCs appeared predominantly at the early stages of fungal growth, whilst others were only observed later. For example, *A. alternata* produced volatiles after two days that were only produced by *A. infectoria* after seven days. Compounds that are produced after two days are potentially strong indicators of the presence of spoilage fungi on grain (Magan and Evans 2000). These results are consistent with Girotti *et al*. (2012) who reported that the early detection of volatiles (intermediates of trichothecenes) of *Fusarium graminarum* in wheat after 48 h. Keshri *et al*. (1998) found that volatiles could be produced by four spoilage fungi (*Eurotium chevalier, E. rubrum, Penicillium* sp. and *Wallemia sebi*) on milled wheat agar media after 48 h. Vinaixa *et al*. (2004) confirmed early detection of *Eurotium, Penicillium* and *Aspergillus* spp. within 48h growth in bakery products. Whilst de Lacy

Costello *et al*. (2003) confirmed that sensor systems can be used to assess the early detection of *A. flavus* in inoculated wheat grain after 3 days. The present study indicated that it was possible to differentiate between species based on the volatile production patterns detected after two days. However, more detailed studies are required on volatiles produced by more fungal species and isolates within a species for the rapid and early detection of fungal spoilage in grain.

The gamma-irradiated grain (controls) did not produce specific volatiles compared to original grain (farm) samples or grain spiked with known fungi. Gamma-irradiated grain could affect the nutrients available for fungal metabolism probably due to of deterioration in both starch and gluten (Siddhuraju *et al*. 2002) causing differences in the volatile profiles. For example, De Lucca (2012) reported that the number of volatiles produced by *A. flavus* isolates grown on autoclaved corn were much lower in number than those produced on the non-autoclaved kernels. Consequently, future work should determine if volatiles produced on gamma-irradiated grain are unique to only gamma-irradiated grain.

It is clear that there are a range of characteristic volatile odours produced by fungi when colonising grain that might be useful for the early detection of grain spoilage. Indeed, several researchers (De Lucca *et al*. 2012; Lippolis *et al*. 2014) recommended that the monitoring for the appearance of volatiles might be a good early indicator of quality loss and mycotoxin formation in grain. As a consequence, there is an absolute need for early and efficient methods to detect infected grain and to distinguish between relevant and harmless species. The priority for the grain industry should be evaluating the occurrence of moulds in Australian grains by means of volatiles produced under different conditions and when various fungal species are present. Developing modern, fast and easy tools for identification of spoilage at early stages will allow significant losses and grain downgrading to be avoided.

Chapter 6: General Discussion

Source: Courtesy of James Newman, Postharvest Laboratory, Murdoch University

General Discussion

The overriding purpose of the work presented in this thesis was a modern investigation of the fungi associated with stored wheat grain in Australia, using a range of methods for isolating, identifying and characterising such fungi. Conditions for mould growth in grain were identified.

Australian grains are globally regarded for their high quality and reliability, both as bulk commodity exports and as value-added products which combined was valued at approximately $22.8 billion in 2013–14 (Sarina 2014). The total amount of wheat grain grown across Australia was 25 million tonnes per year (AEGIC 2015) and WA was the largest wheat grain exporter in Australia. The value of WA grain exports in 2014/2015 was worth over $ 5.1 billion with $3 billion of this value from wheat (Department of Agriculture and Food WA 2016). As a high value export crop, it is important to understand the abiotic (e.g. temperature and moisture) and biotic (microorganisms and insect pests) factors that affect yield loss.

Additionally, there is concern that the industry will be affected by global warming due to changes in seasonal rainfall, extreme climatic events such as droughts, floods, tropical cyclones and severe storms, which can damage stored grain (Yang *et al*. 2014). Once harvested, grain is always influenced by how it is handled and stored, as well as the environmental conditions it is exposed to. The potential sources of contamination during handling and transport are grain residues and dust, which can be heavily contaminated with fungal spores. Further, fungi that infest grain before harvest may further develop during storage if conditions are conducive to fungal growth. Dust from vegetative parts of plants, soil thrown up during harvesting, residues in combine harvesters and storage sacks appear to be possible sources of contamination of grain during harvesting and the spores from these sources can germinate and spoil stored grain (Flannigan 1978). This is important because grain storage is the key link in the grain demand–supply chain.

Briefly, the major findings from this research were:

1. Fungi can be isolated from healthy wheat grain, even if the grain is stored and managed correctly. However, the number of fungi did decline over time (Experiment 2, Chapter 2). This decline in fungi over time reinforces the advantages of using storage facilities for extended periods that are protected against environmental conditions (e.g. moisture and temperature) that are conducive for fungal growth. If this occurs, then the presence of fungi in the grain declines.
2. A wide range of fungi were present on wheat grain and isolated into pure culture, these included *Alternaria, Aspergillus, Aureobasidium, Cladosporium, Drechslera, Eutiarosporella, Fusarium, Mucor, Nigrospora, Penicillium, Rhizopus, Setosphaeria, Stemphylium, Ulocladium* and *Epicoccum* (Chapter 3). Additionally, a new species from the genus *Eutiarosporella* was identified and described (Chapter 4). DNA methods were used to confirm the identification of these isolates together with morphological diagnosis.
3. Diversity profiling of wheat samples collected from on-farm storage facilities identified a large number of fungi that were not isolated using traditional methods. The fungi identified included *Cryptococcus macerans,* which is a known fungal pathogen to humans, and this, amongst other fungi, were never isolated in culture (Chapter 3). This finding highlights the importance of screening grain prior to storage, to determine what fungi (and potentially other microorganisms and insects) are present prior to storage. This will allow grain storage managers to make informed decisions on how individual grain batches might be handled and processed during storage and potentially post-storage. For example, if a storage facility becomes compromised by excess moisture or insect invasion, then the manager will have prior knowledge about which organisms may become active. Alternatively, the microorganisms and/or insect pests could be controlled or managed immediately; for example, application of phosphine for insect control. In addition, diversity profiling of grain could be used as a marketing strategy by grain growers/bulk storage handlers, to indicate the absence of putatively detrimental microorganisms.
4. The Headspace Solid-Phase Microextraction method combined with GC/FID and GC/MS technologies for separation and identification of volatile organic compounds demonstrated that fungi can produce unique VOCs. In this study, one isolate each from

Alternaria alternata, *A. infectoria* and *Cladosporium herbarum* were studied in detail and each were shown to produce specific VOCs. Three specific compounds were identified and included Cyclooctasiloxane, hexadecamethyl-, Pentadecane and Naphthalene which were specific to the headspaces of grain colonized by *Alternaria alternata*, *A. infectoria* and *Cladosporium herbarum*, respectively. Hence, although only a preliminary study, these VOCs can potentially be used to determine the presence of specific spoilage fungi, particularly those that are known to produce mycotoxins in storage silos.

These points and their implications are discussed in more detail below.

Growth and survival of fungi on healthy stored grain over time

The basis of controlled atmospheres for stored grain is to protect postharvest grain from infestation and damage by insects and microorganisms. In this study, the viability of fungi on stored grain started to decline after six months. This decline was likely due to low concentrations of O_2 and the accumulation of high concentrations of CO_2. However, the surface:volume ratio of farm silos is large compared to the jars that were used in the present study, which greatly modifies the stored grain conditions. In future it will be necessary to compare the results from the present study to studies within grain silos under controlled conditions (temperature, moisture). Extending this work under realistic storage conditions, with manipulation of the concentrations of CO_2 or O_2 and increasing the storage period to 14 months (or more) should determine the influence of these gases on the growth of fungi in the long term.

Presence of fungi from different growing regions across Australia

There were significant differences in fungal species isolated across Australian grain growing regions. These regions have a wide range of climatic conditions: the Southern Region (Victoria and South Australia) has a temperate climate, the Western Region (Western Australia) has a Mediterranean climate, and the Northern Region (New South Wales) has a subtropical-temperate climate. Both temperature and humidity influence which fungi infect crops and in turn could be the main reason for the variation of mycoflora

between states observed in this study. The number of fungi isolated from WA grain was the highest, followed by SA, Victoria and NSW. These results concur with Cotty and Jaime-Garcia (2007) who found that crops grown in warm climates have greater likelihood of infection by aflatoxin producers.

Soil is another factor that could influence the presence of fungi associated with stored grain. For example, soils in southern (Victoria and South Australia) and western regions (WA) are poor and low in fertility. Soil moisture and nutrients influence the growth of fungi during different seasons (Burgess *et al*.1975). More extensive surveys over a number of years should be conducted across Australian grain growing regions to understand how fungi vary in postharvest grain with respect to climate (e.g. temperature, rainfall, relative humidity, soils), handling methods and the types of storage facilities used.

The distribution of fungi is likely dependent upon the environmental conditions at the time of harvest in different regions and the difference between the storage structures. Pitt and Hocking (2003) reported the major storage fungi associated with Australian wheat were *Alternaria, Fusarium, Aspergillus* and *Penicillium* and these may form mycotoxins. The present study also isolated into culture species from these genera, in addition to species from another 10 genera. These fungi can develop on plants in the field before they are harvested and could be found in high amounts in storage facilities. Akinsanmi *et al* (2004) indicated that *Fusarium* species associated with head blight (FHB) and crown rot (CR) of wheat were collected from wheat paddocks with different cropping histories in southern Queensland and northern New South Wales during 2001. Smiley *et al*. (1993) demonstrated that the dominant microbial colonists of the leaf spots on wheat were *Cladosporium herbarum, C. macrocarpum, C. cladosporium* and *Alternaria* species. Nicolaisen *et al*. (2014) indicated that the most important pathogens during harvest associated with seeds are *Alternaria, Cladosporium* and *Fusarium.* They also reported that *Penicillium* spp., and *Aspergillus* spp., could be found in high amounts in stored grain if grain was not dried. These fungi require a moisture content in equilibrium with a relative humidity of more than 90% (24-25% moisture content) to grow in wheat and temperatures of 30-33ºC, and they do not continue to grow in grains after harvest, since grains and seeds are stored at moisture contents below those required by the field fungi (Christensen and Kaufmann 1965). There are exceptions for finding these species in storage facilities, these occur when wheat might

stored at high moisture contents and temperatures or if sources of contamination such as dust and soil enter during harvesting. The present study showed that increasing moisture contents increased the occurrence of fungi.

A new species of *Eutiarosporella* was isolated from grain from Cooperative Bulk Handling and from farms in Western Australia, and is described as *E. pseudotritici-australis* sp. nov based on the ITS gene region. This is the first report of this particular *Eutiarosporella* associated with Australian grain. *Eutiarosporella* belongs to the family Botryosphaeriaceae and can cause extensive damage to grain (e.g. white grain) (Thynne *et al*. 2015). It is possible that *Eutiarosporella* can cause further damage postharvest if the temperature and moisture content during storage are conducive to the fungus.

The present study reported that Western Australia and South Australia have suitable environment conditions for the growth of fungi such as *Alternaria* species in comparison to New South Wales and Victoria. An open question here is why the grain from Western Australia contained more fungal contaminants compared to other regions such as grain from VIC, QLD and NSW (when looking at numbers of isolations using traditional methods). It is important to note that WA is more advanced in postharvest storage technologies than the eastern states. For example, sealed silos are common in WA, which should significantly reduce the development of insects or fungi during storage (Andrews *et al*. 1994). Therefore, it was unexpected to find more fungal contaminants in WA than elsewhere in Australia. The reasonable explanation for this finding is due to the treatments (pre- and postharvest) and atmospheric conditions used in the silo,that could help to increase fungal growth during storage time. A prevention strategy to minimise the occurrence of contamination is to ensure that grain is effectively dried immediately after harvest. Often, air-drying using heat may cause an increase in moisture content (post heating) that is sufficient to shorten its storage life considerably (Jian and Jayas 2012). If hot grain is allowed to cool naturally, the relative humidity of the air in the bin will increase, and if the saturation temperature is reached or exceeded, condensation can cause the grain moisture content to increase again (Magan *et al*.2010). When the moisture content is increased, fungi if present can colonise the grain and begin to decompose it if conditions remain conducive (Lacey *et al*. 1996; Magan *et al*. 2008).

Diversity Profiling

The diversity profiling analysis of farm samples resulted in very different numbers of potential fungal contaminants (58 species) compared to traditional methods (23 species); although the results still confirmed the variation between growing regions in the distribution of these species. The identification of *C. macerans, C. oeirensis* and *C. victoria* on Australian grain, albeit using molecular methods, appears to be the first record of these pathogens being present in stored wheat in Australia. *Cryptococcus* spp., are considered serious human pathogens (e.g. causing Meningoencephalitis diseases) Lindsberg *et al*. (1997). There is concern that rain-splash, wind, insects and poor hygiene management may spread these pathogens during transport and storage. Their presence in grain may pose a threat to human and animal health in the future, if they become more prevalent in postharvest grain. Their presence could be further exacerbated if storage conditions are sub-optimal and allow these fungi to colonise grain more extensively. It is important to note these species were not isolated by traditional methods in the present study. To confirm how frequently members of the *Cryptococcus* are presence on grain, it would be useful to extensively sample grain from across Australia, specifically targeting *Cryptococcus* spp and other fungi that were not isolated in culture. This in turn would help inform mangers and policy makers about the potential risk of these fungi to humans and animals, and in turn trigger appropriate management strategies. The use of diversity profiling as a diagnostic and monitoring tool has huge potential in the future for ensuring food security and food safety, both domestically and internationally.

Detection of postharvest fungi on wheat grain using volatiles

The *in vitro* studies (Chapter 5) where individual isolates were inoculated onto gamma irradiated grain, showed that specific VOCs could be produced by different fungal species. However, none of these volatiles were produced in the present study from the bulk grain samples, which may partially be due to the differences in the nutrient substrate used for the culturing of the fungi (e.g. gamma-irradiated grain versus non-irradiated grain) or probably due to different exposure times prior to analysing the VOCs. For example, Paolesse *et al*. (2006) showed that many factors such as substrate, temperature, pH, oxygen concentration,

age of culture on wheat and microbial species can influence the composition of volatiles. These findings suggest more research is required to further examine the possibilities of monitoring these factors (e.g. O_2, temperature, species of fungi and time) on the production of volatile fungal metabolites. Further research should also focus on detecting individual fungal species as well as the amount of volatiles produced on grain. More testing using greater numbers of species, and isolates of the same species, is required to confirm if specific volatiles are produced by individual fungal species or alternatively can be produced by a range of fungal species. Finally, it would be appropriate to determine if the fibres used in the present study can be inserted inside silos and left for various periods of time, before being processed by HS-SPME together with GC/FID and GC/MS to accurately monitor VOCs produced by storage fungi. In addition, the fibres could be used to detect VOCs in other postharvest commodities such as cut flowers, fruit and vegetables, and to monitor for pathogens. Diversity profiling and VOCs profiles are both potentially useful tools to confirm the presence of human pathogens such as *Cryptococcus macerans* and *Cryptococcus victoriae* at an early stage of contamination of grain during storage.

Future directions

This study has improved our knowledge of postharvest problems in stored wheeat grain in Australia, and has laid a foundation for future research into several aspects of the isolation and identification of fungi on stored grain. The following points address possible future research directions:

1. Expand on the biological significance of environmental conditions on growth of fungi for long-term storage. Factors that should be addressed include the control of CO_2 and O_2 levels and how these might interact with temperature and moisture content together over the longer term (12-24 months) with regards to fungal survival, growth and VOC production. Such studies could use traditional isolation techniques, molecular methods, and the measurement of VOCs in the headspace of containers/silos to monitor fungal growth and survival.

2. The main postharvest storage insects include *Tribolium castaneum* (Phillips *et al*. 1993; Niu *et al*. 2012), and *Rhyzopertha dominica* (Niu *et al*. 2012), and these have been shown to produce specific VOCs whilst feeding on grain in postharvest facilities (Phillips *et al*.

1993; Niu *et al*. 2012). Neethirajan *et al*. (2007) showed that there is a strong interaction between fungi and insects in stored grain. Consequently, it would be useful to expand the volatile studies to include volatiles produced during interactions between insects and postharvest fungi. This would indicate if the volatiles are specific and/ or unique to particular fungal species in the presence of insect pests or whether other specific VOCs are produced with fungi are present with particular insect pests. Such studies would allow postharvest storage managers to determine whether only insects were present, only fungi present or a combination of both. In turn providing managers with the knowledge of what control measures are necessary.

3. It would also be beneficial to use a combination of diversity profiling together with the detection of specific VOCs known to be associated with mycotoxin production, or VOCs produced by specific fungal species known to produce toxins (Sahgal *et al*. 2007). The use of diversity profiling will provide detailed insights into what fungi are present in a particular grain storage facility, whilst VOC detection will indicate if conditions in the facility are sub-optimal and allowing the fungi present to proliferate and produce mycotoxins or other spoilage compounds.

In conclusion, this study has highlighted the potential benefits of using both traditional and molecular methods together with VOC detection, to monitor the presence of specific fungi in postharvest storage facilities.

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Appendix 1

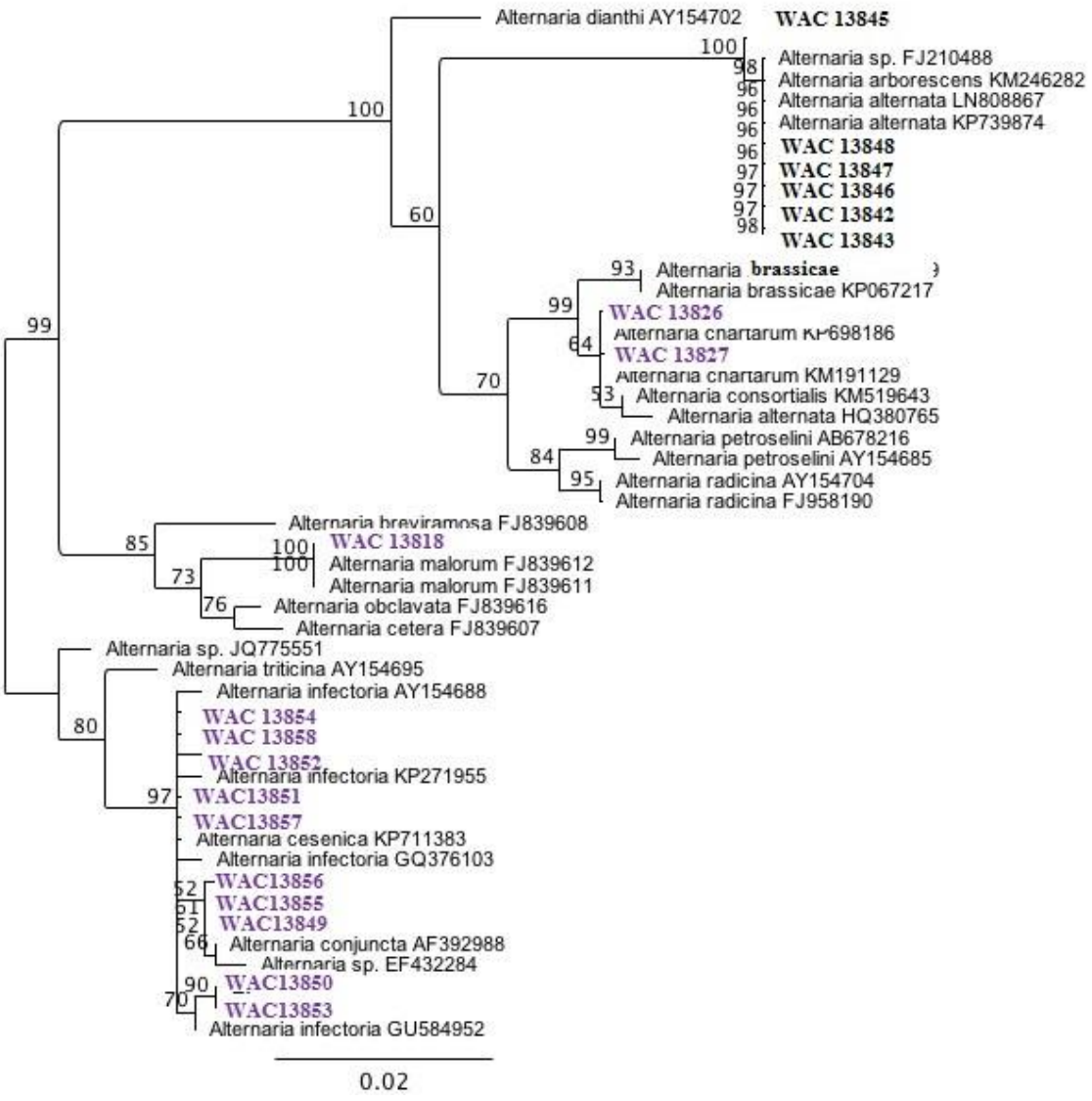

Fig. A1 Comparison of ITS sequences (Table 2) based on culture morphology showed that seven of the *Alternaria* isolates appeared to be most closely related to *A. alternata* (WAC 13842, WAC 13843, WAC 13844, WAC 13845, WAC 13846, WAC 13847, WAC 13848), two related to *A. chatarum* (WAC 13826, WAC 13827), one belonged to *A. malorum* (WAC 13818) and ten (WAC13849, WAC13850, WAC13851, WAC 13852, WAC13853, WAC 13854, WAC13855, WAC13856, WAC13857, WAC 13858) to the *A. infectoria* clade.

Appendix 2

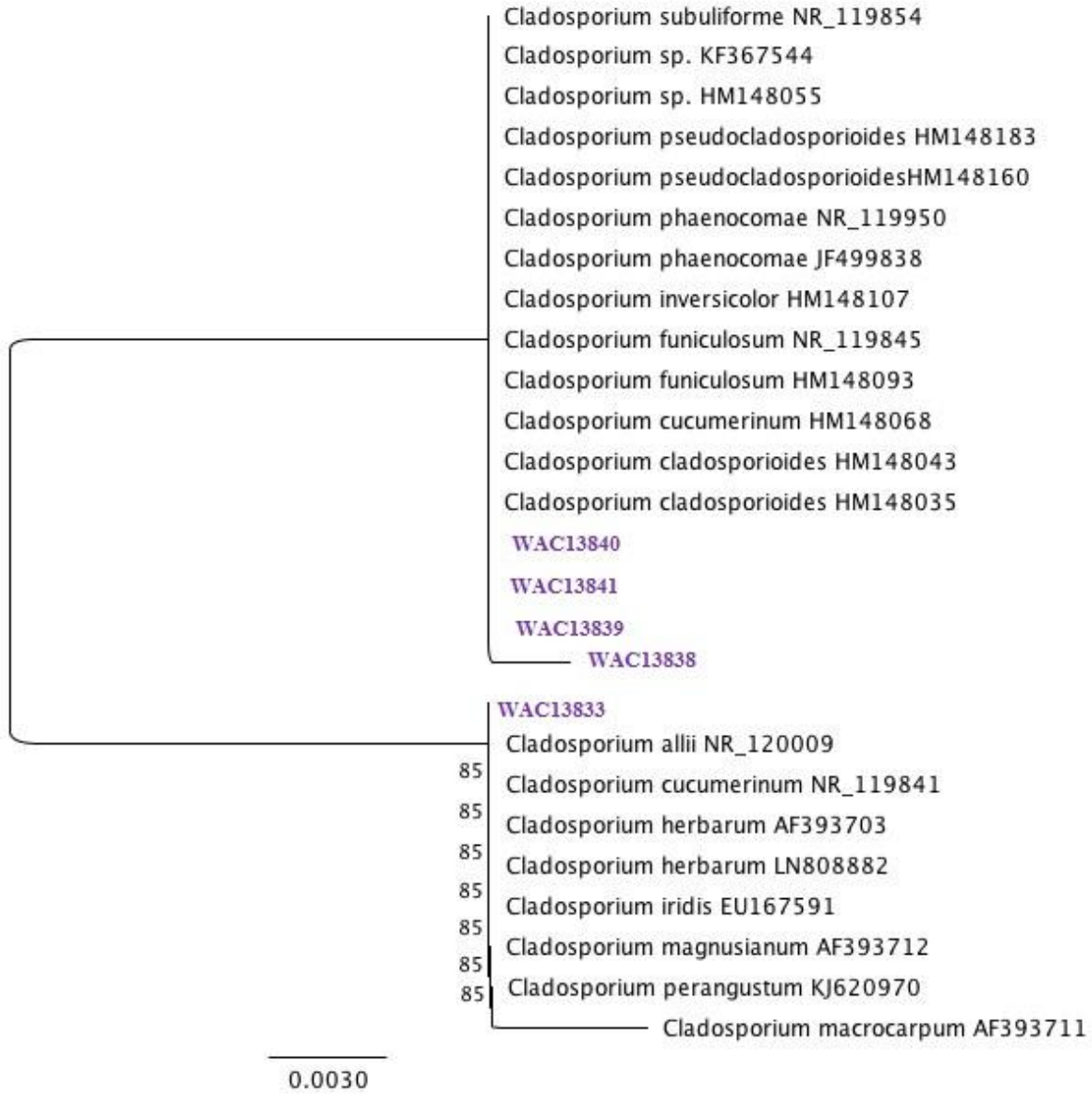

Fig. A2 Based on sequence data and phylogenetic analysis of ITS sequences from Table 2 showed that four isolates were closely related to *Cladosporium cladosporioides* (WAC13840, WAC13841, WAC13838, WAC13839), and one isolate to *Cladosporium herbarum* (WAC13833).

Appendix 3

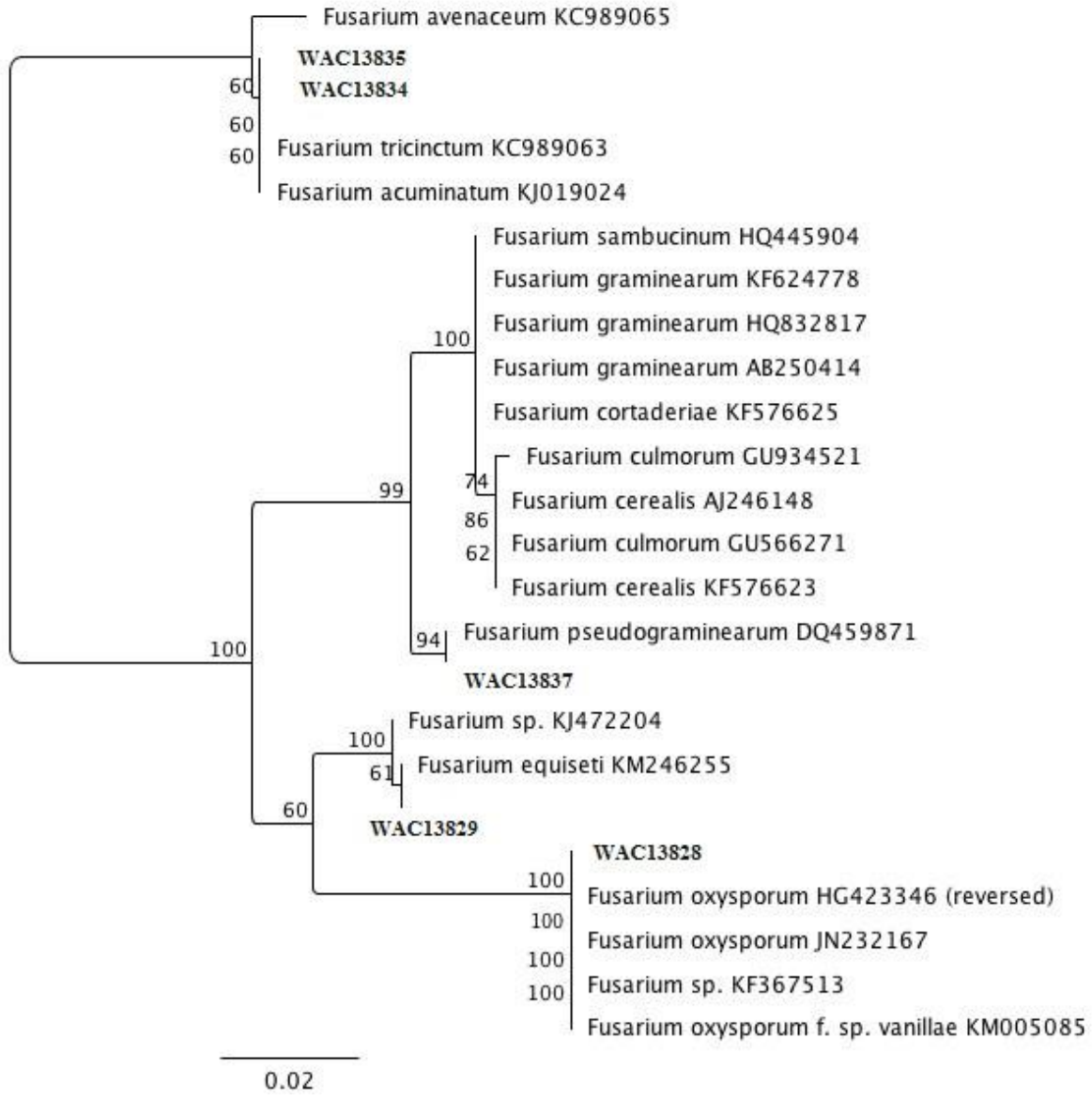

Fig. A3 Based on sequence data and phylogenetic analysis of ITS sequences (Table 2) showed that two isolates of *Fusarium* were closely related to *Fusarium tricintum* (WAC13834, WAC13835) and one each to *Fusarium pseudograminearium* (WAC13837), *Fusarium equiseti* (WAC13829) and *Fusarium oxysporum* (WAC13828).

Appendix 4

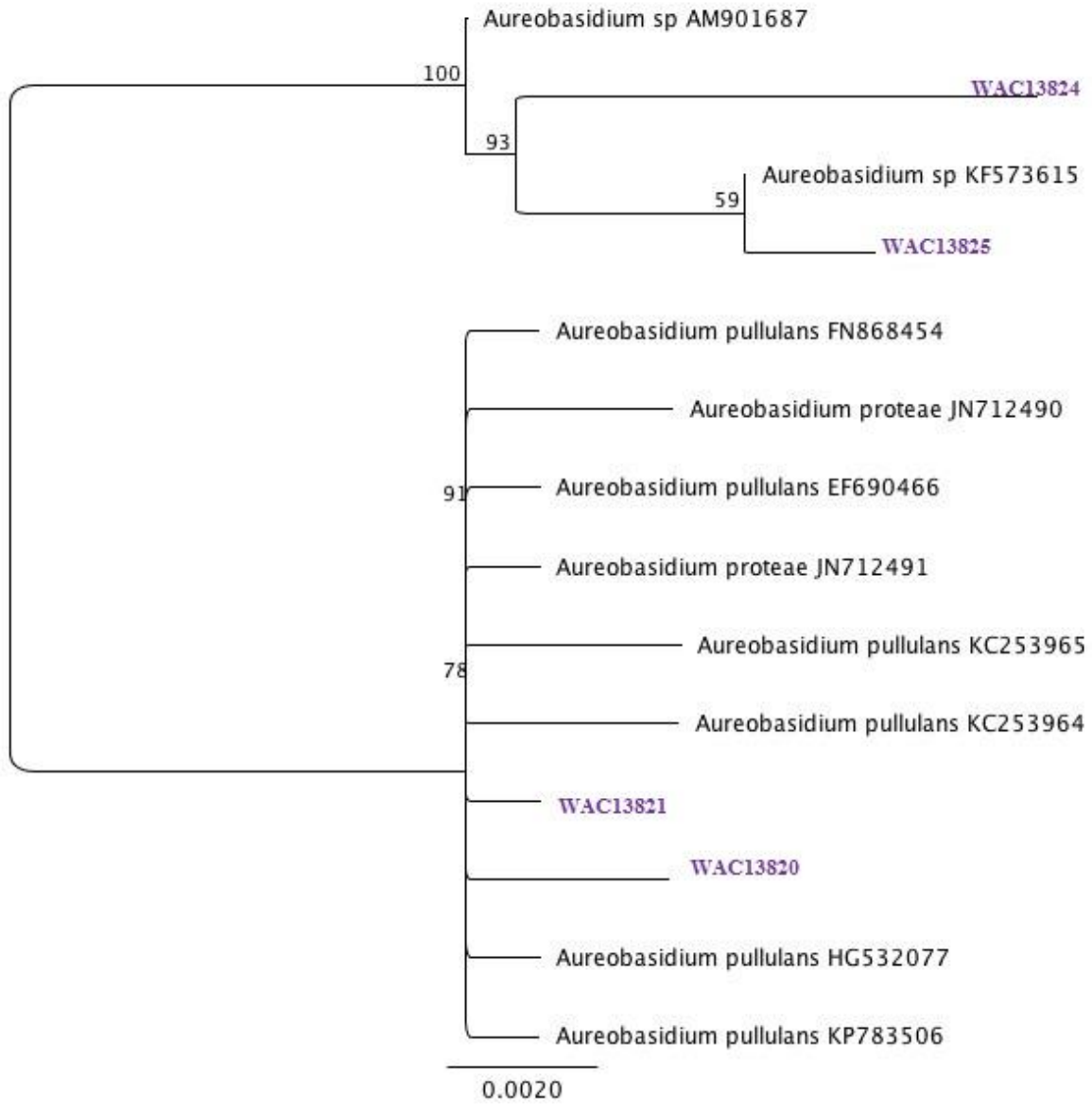

Fig. A4 Comparison of ITS sequences from Table 2 showed that two isolates of *Aureobasidium* aligned to *Aureobasidium pullulans* (WAC13820, WAC13821) and two isolates appeared to be supposed new *Aureobasidium* species (WAC13824, WAC13825).

Appendix 5

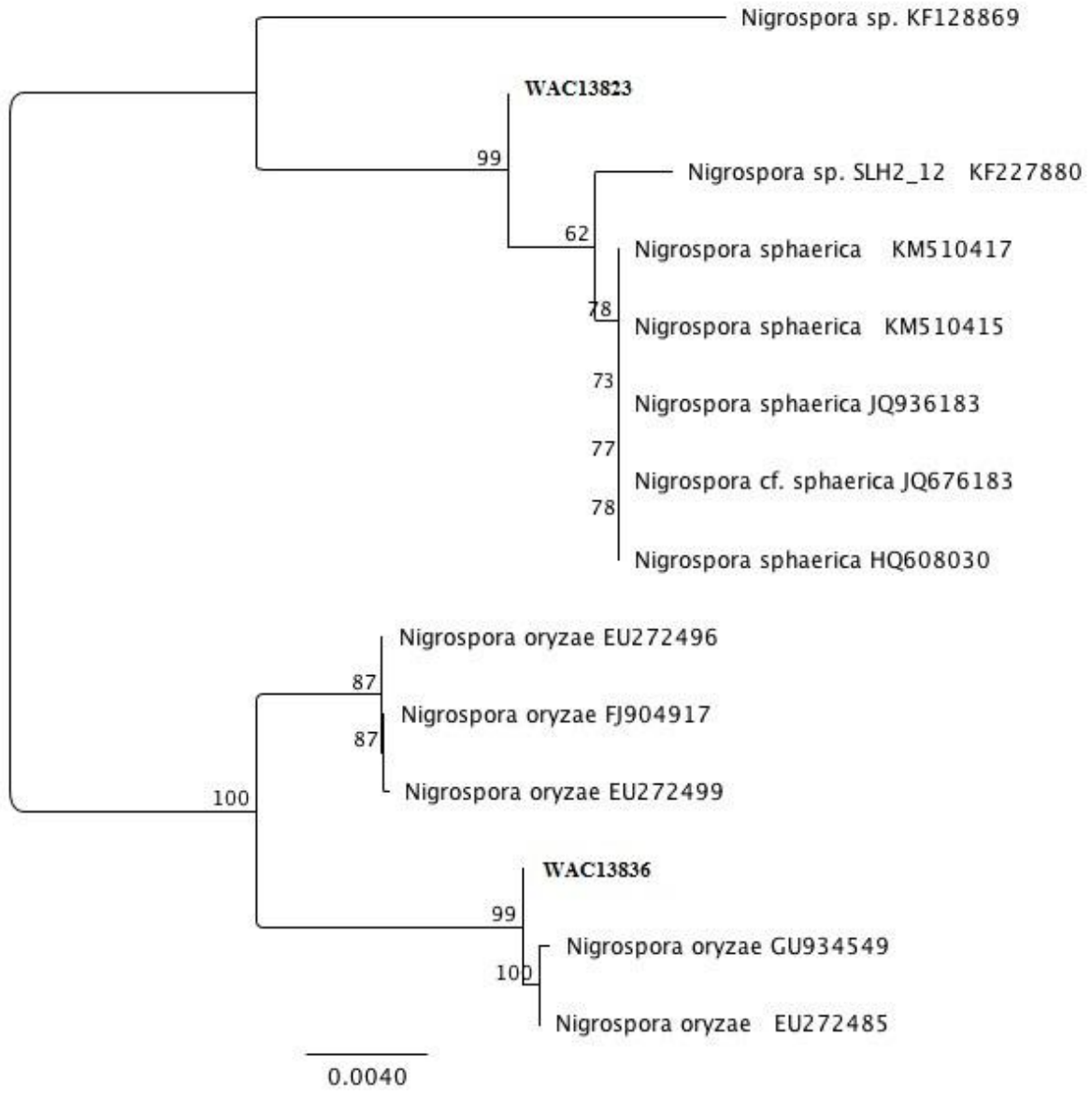

Fig. A5 The phylogenetic analyses based on ITS sequences from Table 2 showed that one isolate was close to *Nigrospora oryzae* (WAC13836) and another appears to be an unidentified *Nigrospora* sp (WAC13823).

Appendix 6

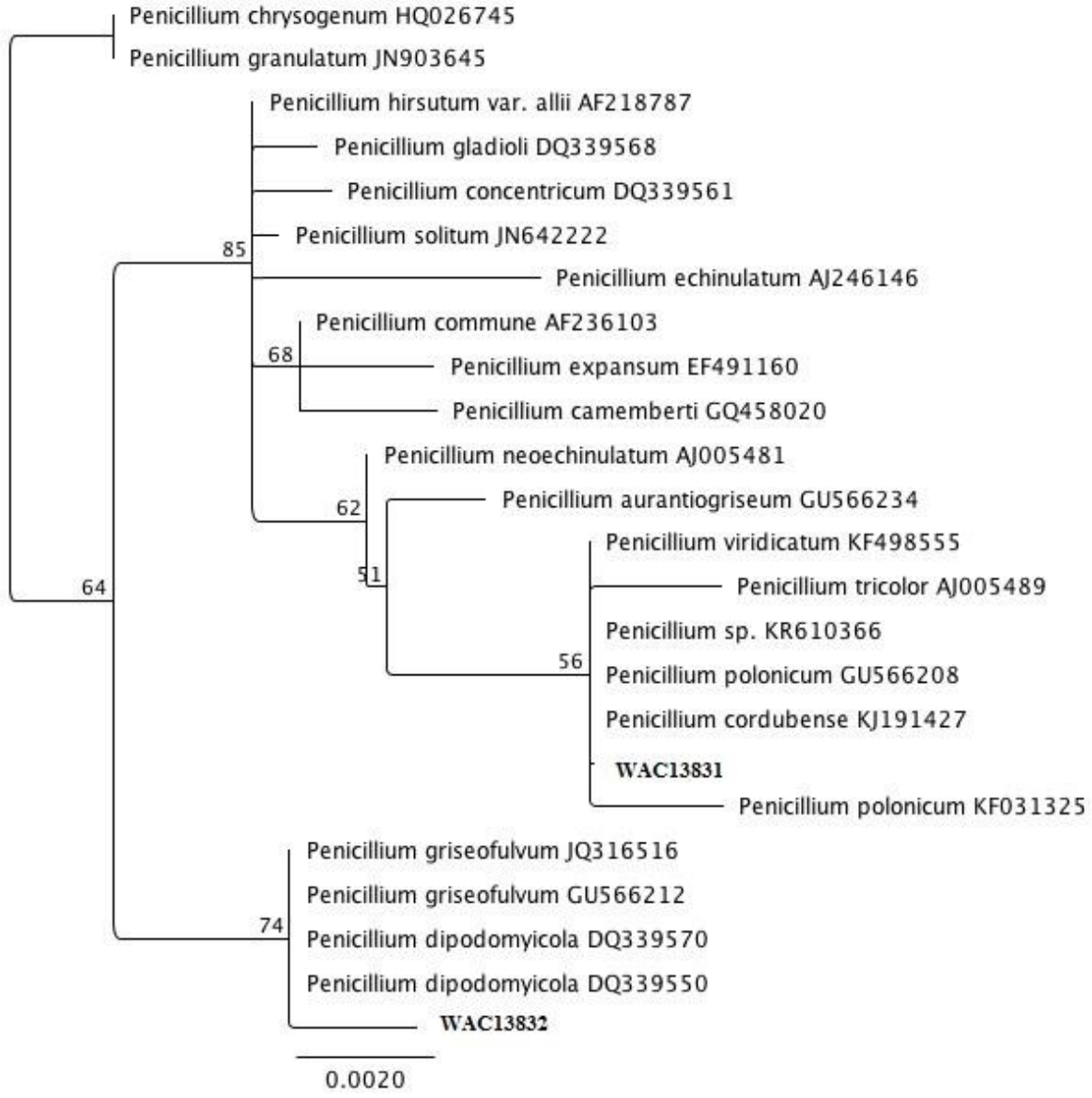

Fig. A6 Based on sequence data and phylogenetic analysis in Table 2 one isolate was closely related to *Penicillum cordubense* (WAC13831) and another isolate close to *P. dipodomyicola* (WAC13832).

Appendix7

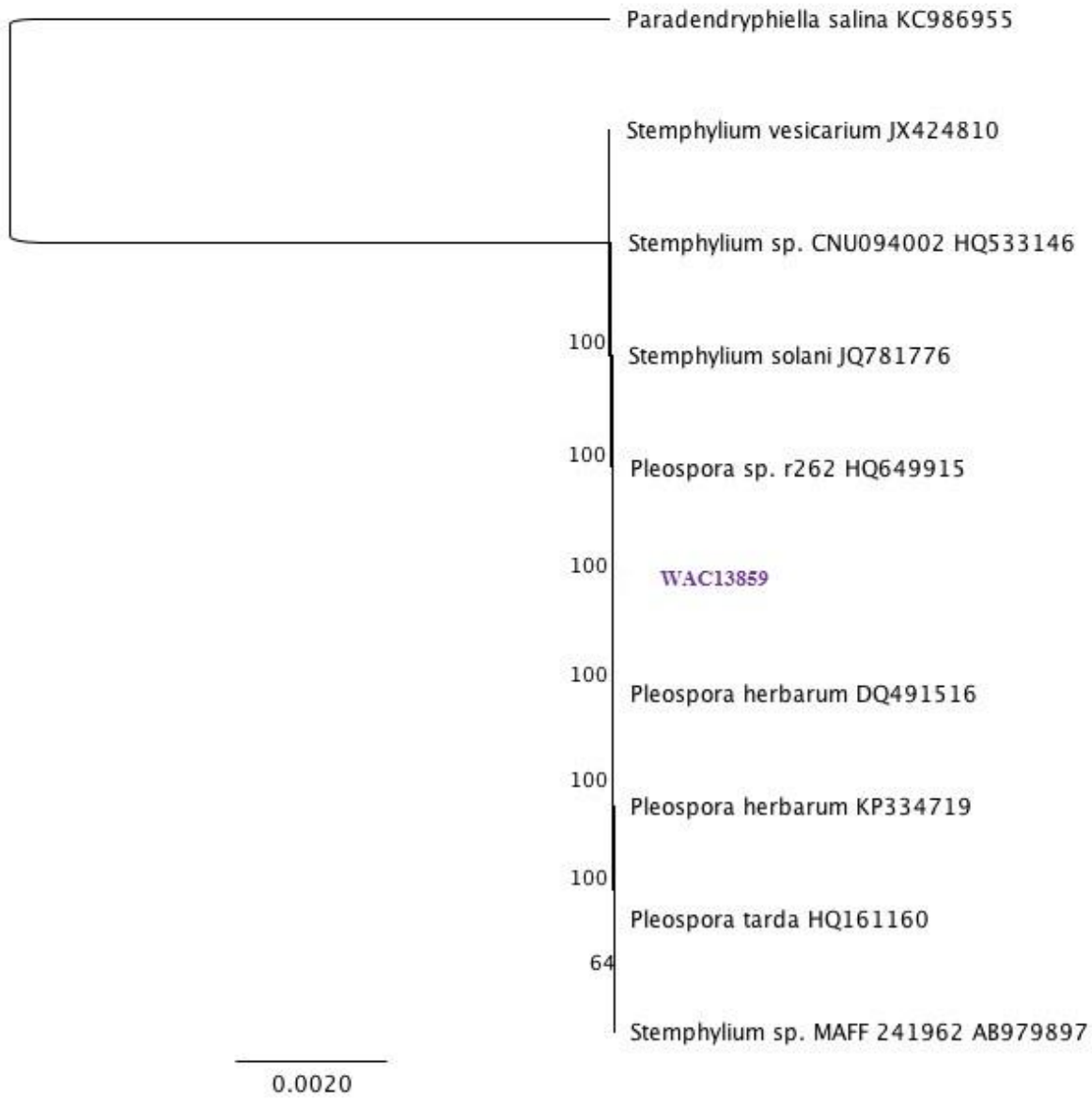

Fig. A7 Comparison of sequence data and phylogenetic analysis from Table 2 confirmed one isolate of *Stemphylium* (WAC13859) appeared to be an undescribed species.

Appendix 8

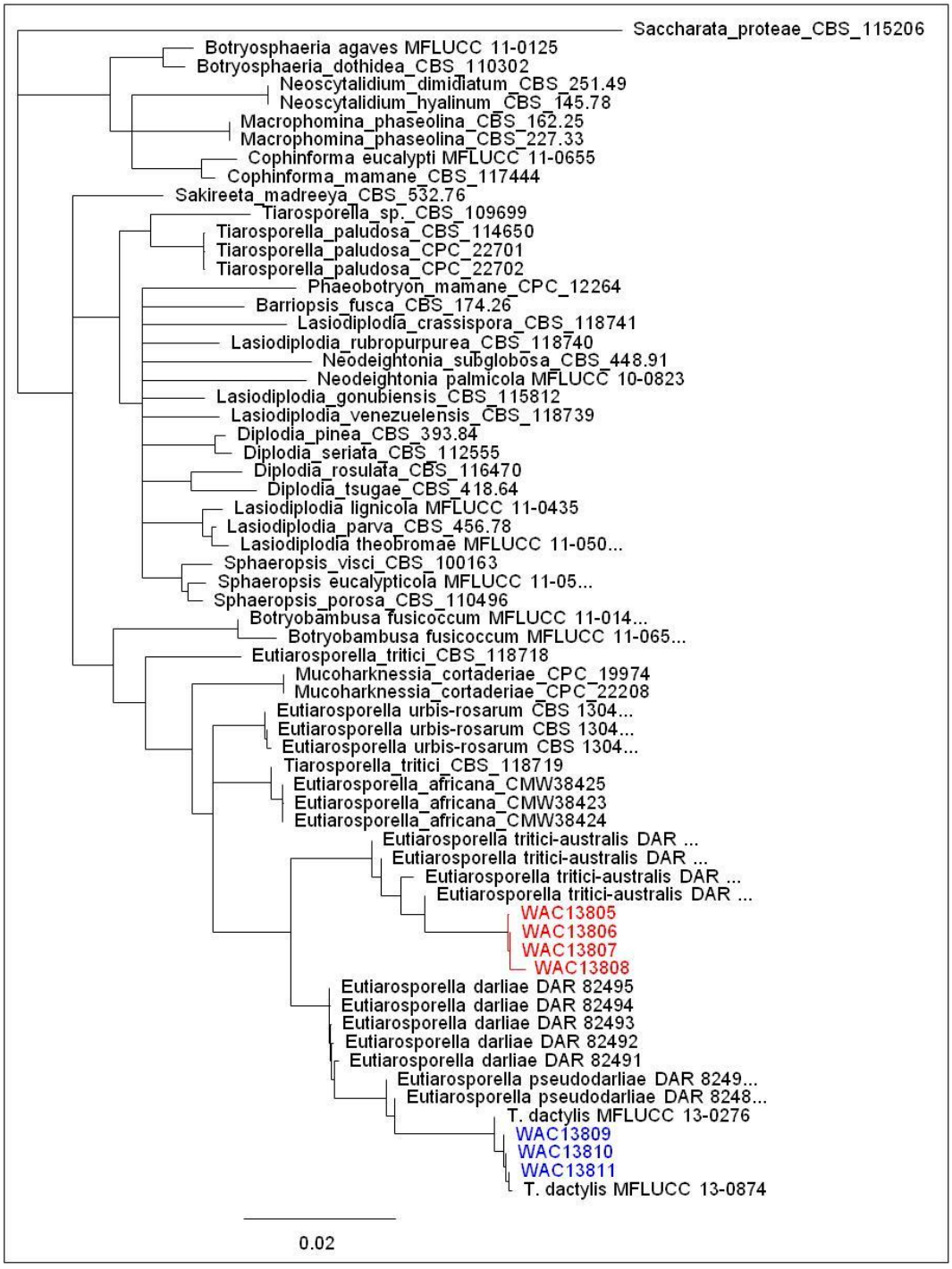

Fig. A8 Sequence data and phylogenetic analysis confirmed that four isolates were *Eutiarosporella pseudotritici-australis* sp. nov. and three isolates were *E. dactylis*.